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(54) Title: HUMAN INTERLEUKIN-13

(57) Abstract

Nucleic acids encoding human IL-13, and purified IL-13 proteins and fragments thereof. Antibodies, both polyclonal and monoclonal, are also provided. Methods of using the compositions for both diagnostic and therapeutic utilities are provided.

HUMAN INTERLEUKIN-13

The present invention relates to compositions and methods for affecting the human immune system.   In particular, it provides
5   nucleic acids, proteins, and antibodies which regulate immune system response and development.   Diagnostic and therapeutic uses of these materials are also disclosed.

BACKGROUND OF THE INVENTION

For some time, it has been known that the mammalian
10   immune response is based on a series of complex cellular interactions, called the "immune network."   Recent research has provided new insights into the inner workings of this network.

While it remains clear that much of the response involves network-like interactions of lymphocytes, macrophages,
15   granulocytes, and other cells, immunologists now generally hold the opinion that soluble proteins, known as lymphokines, cytokines, or monokines, play a critical role in controlling these cellular interactions.

In view of their importance, there is a need to identify and
20   isolate new lymphokines.

SUMMARY OF THE INVENTION

The present invention fills this need by providing one such new lymphokine.   More particularly, this invention provides human interleukin-13 (IL-13), and methods for its use.
25   This invention also provides nucleic acids coding for polypeptides themselves and methods for their production and use. The nucleic acids of the invention are characterized by their homology to cloned complementary DNA (cDNA) sequences enclosed herein, and/or by functional assays for IL-13 activity applied to the
30   polypeptides, which are typically encoded by these nucleic acids.

Methods for modulating or intervening in the control of an immune
response are provided.

The invention is based, in part, on the discovery and cloning of
human cDNAs which are capable of expressing proteins having
IL-13 activity. cDNA clones include human cDNA inserts of plasmid
vectors pB21.2Bf and pA10.66, which contain partial and full length
sequences, respectively. Equivalent vectors may be constructed by
using polymerase chain reaction (PCR) techniques and the sequences
of the inserts.

The present invention provides an isolated nucleic acid
comprising a segment homologous to a sequence of human IL-13
disclosed in Table 1. Typically, the segment is at least about 50
nucleotides, and will often encode a protein exhibiting a biological
activity characteristic of a human IL-13, e.g., an amino acid
sequence of Table 1. In other embodiments, the segment is at least
80% homologous to the coding sequence disclosed in Table 1. In
other embodiments, the nucleic acid will further encode a second
protein. The invention also encompasses a vector or a cell
containing the nucleic acid.

Alternatively, the nucleic acid can be a recombinant nucleic
acid with a segment homologous to a sequence of human IL-13
disclosed in Table 1. Usually, this will encode a human IL-13 or
may encode a fusion protein. The invention also embraces vectors,
e.g., expression vectors, and cells containing the nucleic acid.

In alternative embodiments, the invention provides an
isolated human IL-13 protein or peptide. In some embodiments, the
protein has a full length sequence disclosed in Table 1, or will be a
mutein thereof, and may include an altered post-translational
modification pattern, e.g., glycosylation variants. Other
embodiments include a fusion protein comprising a peptide of
human IL-13, and cells containing such.

In another embodiment, the invention provides a method of
refolding a guanidine denatured mouse P600 or human IL-13
protein comprising solubilizing said protein in 6M guanidine at a
concentration of about 2.5 mg/ml; diluting the guanidine to about 60
mM over a period of hours in the presence of both reduced and

oxidized glutathione; and incubating the diluted guanidine solution for at least about 12 hrs.

The invention also provides an antibody which specifically binds to human IL-13, e.g., a mouse, a monoclonal, or a chimeric antibody. It also provides a method of supporting monocyte or B cell proliferation in a sample, or sustaining viability of said cell, by contacting the sample with an effective amount of human IL-13, alone or in combination with another cytokine, e.g., IL-4 or IL-10. In some embodiments, methods are provided for detecting human IL-13 in a sample by contacting the sample with a binding composition which recognizes human IL-13 or a nucleic acid which hybridizes to a nucleic acid encoding a human IL-13. The binding composition can be a monoclonal antibody, and the sample can be a blood sample.

In other embodiments, the invention provides methods of modulating the growth of a hemopoietic B cell or T cell by contacting the cell with an effective amount of an IL-13 and IL-4 combination or antagonists thereto, including an IL-4 antagonist. The hemopoietic cell growth can be accompanied by cell differentiation to antibody producing cells.

The invention further provides methods of modulating proliferation of a myeloid precursor cell by contacting the cell with an effective amount of a human IL-13, mouse P600, or agonists or antagonists thereof. Often the modulating proliferation is accompanied by cell differentiation.

Methods of modulating the immune response to an infection or allergen are provided, e.g., by administering an effective amount of a human IL-13, mouse P600, or agonists or antagonists thereof, including an IL-4 antagonist. And the invention provides methods of sustaining cell viability of a myeloid precursor cell by contacting the cell with an effective amount of a human IL-13, a mouse P600, or an agonist or antagonist thereof, including an IL-4 antagonist, and combinations with additional cytokines, including IL-4 and IL-10.

DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

I. General

5   The present invention provides the amino acid sequence and DNA sequence of human interleukin molecules having particular defined properties, both structural and biological, designated herein as human interleukin-13 (IL-13). This molecule was obtained using a mouse gene encoding a related mouse protein designated P600.

10   Some of the standard methods are described or referenced, e.g., in Maniatis *et al.* (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor Press; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, (2d ed.), vols 1-3, CSH Press, NY; Ausubel *et al., Biology*, Greene

15   Publishing Associates, Brooklyn, NY; or Ausubel et al. (1987 and periodic supplements) *Current Protocols in Molecular Biology*, Greene/Wiley, New York.

Isolation of the human gene presented obstacles which prevented others from succeeding. Earlier attempts to isolate the

20   human homolog of the mouse P600 protein using oligonucleotide probes and primers ended in failure. Difficulties in using such methods often arise from the inability to select probes of sufficient length which provide a sufficient signal to noise ratio to allow isolation of correct clones. Moreover, many mouse cell lines fail to

25   produce detectable levels of mRNA for the mouse gene, even using highly sensitive polymerase chain reaction (PCR) techniques.

Because the homology of the mouse and human genes is relatively low, about 60%, relatively long probes are needed to provide sufficiently high homology to assure a discernable positive

30   signal by hybridization. But before isolation of the human gene, it was impossible to know the degree of homology or to predict which regions of the target gene exhibit high homology from which the probe should be selected. In fact, multiple attempts using various

probes, alone or in combination, to isolate the gene from various
libraries ended in failure.

The library from which an intermediate clone of less than full
length was isolated failed to provide the correct clone when

5   screened with oligonucleotide or genomic DNA probes. In fact,
clones isolated using the genomic mouse sequence as a probe turned
out to be false positives, i.e., they did not encode the human
equivalent as evaluated by sequencing. At least one other research
group also failed to isolate the gene using a similar approach.

10   A different approach was devised which successfully led to
isolating a human homolog to the mouse P600 gene. Instead of
using oligonucleotide probes of relatively short length, a probe
corresponding to nearly the full length coding region of the mouse
gene was used. Moreover, the cell type used to make the cDNA

15   library was quite important. As indicated above, expression of the
mouse gene varied dramatically in different cell types. The human
B21 cells used to produce the cDNA library which provided the
positive clone described herein turn out to be a cell type which
expresses the human gene at a relatively high level.

20   However, this fact was not apparent when the earlier
screening was performed. In addition, the positive signal arising
from the hybridization was difficult to distinguish from background.
The hybridization and wash conditions used in the screening were
quite important, and slightly more harsh wash conditions could have

25   easily eliminated any positive signal. See, e.g., Wetmur et al., J.
Molecular Biology 31:349 (1968).

The initially isolated clone, designated pB21.Bf2, was less than
full length. Isolating a full length clone required use of yet another
cDNA library. Thus, the isolation of the full length human clone,

30   designated pA10.66 required investment of significant time and
resources. After knowing the regions of high homology between the
mouse and human genes, isolation using oligonucleotide probes of
relatively short length would now be relatively straightforward.

The procedure used to isolate the human IL-13 is broadly set

35   forth below. A cDNA library, constructed in a pCD vector, was
prepared from RNA isolated from human B21 cells. These cells are

human T cells which, it is now understood, exhibit many of the same markers as the cells providing the mouse clone. Several modifications and unusual techniques were utilized to overcome problems associated with isolating a cDNA clone when probing the

5   library with oligonucleotides.

In particular, instead of using oligonucleotide probes of relatively short length, a near full length double stranded probe of about 400 nucleotides was selected. Although previous attempts using a B21 derived cDNA library had failed, the near full length

10   double stranded probe provided faint positive signals. Although several experienced molecular biologists were highly skeptical that the faint signals were real, continued pursuit of those signals led to ultimate success.

The initial human isolate showed homology to the mouse gene,

15   but lacked part of the amino terminal coding portion. Thus, this intermediate isolate was less than a full length clone. Attempts to isolate a full length clone from the B21 derived library failed. However, upon selection of another cDNA library, the near full length human probe allowed isolation of the full length human clone.

20   A complete nucleotide and deduced amino acid sequence of the pA10.66 clone is shown in Table 1. This nucleotide sequence corresponds to the sequence defined by SEQ ID NO: 1. Table 2 compares the gene sequence of Table 1 to a published gene sequence of the mouse P600 protein. Table 3 compares the deduced

25   amino acid sequence of the human IL-13 and the published mouse P600 amino acid sequence.

Table 1:   Nucleotide and Amino Acid Sequence of .huIL-13.

```
 5   TTCGGCATCC GCTCCTCAAT CCTCTCCTGT TGGCACTGGG CCTC ATG GCG CTT TTG   56
                                                      Met Ala Leu Leu
                                                       1

10   TTG ACC ACG GTC ATT GCT CTC ACT TGC CTT GGC GGC TTT GCC TCC CCA   104
     Leu Thr Thr Val Ile Ala Leu Thr Cys Leu Gly Gly Phe Ala Ser Pro
       5               10               15               20

     GGC CCT GTG CCT CCC TCT ACA GCC CTC AGG GAG CTC ATT GAG GAG CTG   152
     Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Glu Leu Ile Glu Glu Leu
                   25               30               35
15

     GTC AAC ATC ACC CAG AAC CAG AAG GCT CCG CTC TGC AAT GGC AGC ATG   200
     Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly Ser Met
                   40               45               50

20   GTA TGG AGC ATC AAC CTG ACA GCT GGC ATG TAC TGT GCA GCC CTG GAA   248
     Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala Leu Glu
                   55               60               65

     TCC CTG ATC AAC GTG TCA GGC TGC AGT GCC ATC GAG AAG ACC CAG AGG   296
25   Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr Gln Arg
           70               75               80

     ATG CTG AGC GGA TTC TGC CCG CAC AAG GTC TCA GCT GGG CAG TTT TCC   344
     Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln Phe Ser
30    85               90               95              100

     AGC TTG CAT GTC CGA GAC ACC AAA ATC GAG GTG GCC CAG TTT GTA AAG   392
     Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe Val Lys
                   105              110              115
35

     GAC CTG CTC TTA CAT TTA AAG AAA CTT TTT CGC GAG GGA CGG TTC AAC   440
     Asp Leu Leu Leu His Leu Lys Lys Leu Phe Arg Glu Gly Arg Phe Asn
                   120              125              130

40   TGAAACTTCG AAAGCATCAT TATTTGCAGA GACAGGACCT GACTATTGAA GTTGCAGATT   500

     CATTTTTCTT TCTGATGTCA AAAATGTCTT GGGTAGGCGG GAAGGAGGGT TAGGGAGGGG   560

     TAAAATTCCT TAGCTTAGAC CTCAGCCTGT GCTGCCCGTC TTCAGCCTAG CCGACCTCAG   620
45

     CCTTCCCCTT GCCCAGGGCT CAGCCTGGTG GGCCTCCTCT GTCCAGGGCC CTGAGCTCGG   680

     TGGACCCAGG GATGACATGT CCCTACACCC CTCCCCTGCC CTAGAGCACA CTGTAGCATT   740

50   ACAGTGGGTG CCCCCCTTGC CAGACATGTG GTGGGACAGG GACCCACTTC ACACACAGGC   800

     AACTGAGGCA GACAGCAGCT CAGGCACACT TCTTCTTGGT CTTATTTATT ATTGTGTGTT   860
```

```
    ATTTAAATGA GTGTGTTTGT CACCGTTGGG GATTGGGGAA GACTGTGGCT GCTGGCACTT  920

    GGAGCCAAGG GTTCAGAGAC TCAGGGCCCC AGCACTAAAG CAGTGGACCC CAGGAGTCCC  980

5   TGGTAATAAG TACTGTGTAC AGAATTCTGC TACCTCACTG GGGTCCTGGG GCCTCGGAGC  1040

    CTCATCCGAG GCAGGGTCAG GAGAGGGGCA GAACAGCCGC TCCTGTCTGC CAGCCAGCAG  1100

    CCAGCTCTCA GCCAACGAGT AATTTATTGT TTTCCTCGT ATTTAAATAT TAAATATGTT  1160
10
    AGCAAAGAGT TAATATATAG AAGGGTACCT TGAACACTGG GGGAGGGGAC ATTGAACAAG  1220

    TTGTTTCATT GACTATCAAA CTGAAGCCAG AAATAAAGTT GGTGACAGAT AAAAAAAAAA  1280

15  AAAAAAAAAA                                                        1290
```

Table 2: Comparison of Human IL-13 and Mouse P600 Nucleic Acid
Sequences (human above; mouse below).

```
 5
                                            TTCGG CATCCGCTCC TCAATCCTCT    25
                                                        I        I I I
            GACAAGCCAG CAGCCTAGGC CAGCCCACAG TTCTACAGCT CCCTGGTTCT    50

10
            CCTGTTGGCA CTGGGCCTCA TGGCGCTTTT GTTGACCACG GTCATTGCTC    75
            I    IIII  IIIIII III IIIIIII I I IIII  I  III I IIII
            CTCACTGGCT CTGGGCTTCA TGGCGCTCTG GGTGACTGCA GTCCTGGCTC   100

15
            TCACTTGCCT TGGCGGCTTT GCCTCCCCAG GCC------- -----CTGTG   113
            I IIIIIII III II I  III IIIIII I I          IIIII
            TTGCTTGCCT TGGTGGTCTC GCCGCCCCAG GGCCGGTGCC AAGATCTGTG   150

20
            CCTCCCTCTA CAGCCCTCAG GGAGCTCATT GAGGAGCTGG TCAACATCAC   163
            III I II       IIII I IIIIII III IIIIIIIII  IIIIIIIII
            TCTCTCCCTC TGACCCTTAA GGAGCTTATT GAGGAGCTGA GCAACATCAC   200

25
            CCAGAACCAG AAGGCTCCGC TCTGCAATGG CAGCATGGTA TGGAGCATCA   213
            II  IIIII I   IIII I I IIIII II IIIIIIIII IIIII  I
            ACAAGACCAG A---CTCCCC TGTGCAACGG CAGCATGGTA TGGAGTGTGG   247

30
            ACCTGACAGC TGGCATGTAC TGTGCAGCCC TGGAATCCCT GATCAACGTG   263
            IIIII I II IIII  II I IIII IIIII IIII IIIII II IIII I
            ACCTGGCCGC TGGCGGGTTC TGTGTAGCCC TGGATTCCCT GACCAACATC   297

35
            TCAGGCTGCA GTGCCATCGA GAAGACCCAG AGGATGCTGA GCGGATTCTG   313
            II    IIII IIIIIII I I IIIIIII IIIII II   II IIII
            TCCAATTGCA ATGCCATCTA CAGGACCCAG AGGATATTGC ATGGCCTCTG   347

40
            CCCGCACAAG GTCTCAGCTG GGCAGTTTTC CAGCTTGCAT GTCCGAGACA   363
             I IIII I I  II     I I II IIII        III  II I
            TAACCGCAAG GCCCCCACT- --ACGGTCTC CAGC------ CTCCCGATA   388

45
            CCAAAATCGA GGTGGCCCAG TTTGTAAAGG ACCTGCTCTT ACATTTAAAG   413
            IIIIIIIIII II IIIIII III III I IIIIII  I    IIII
            CCAAAATCGA AGTAGCCCAC TTTATAACAA AACTGCTCAG CTACACAAAG   438

50
            AAACTTTTTC GCGAGGGACG GTTCAACTGA AACTTCGAAA GCATCATTAT   463
            IIII IIII II I II I  III I III
            CAACTGTTTC GCCACGGCCC CTTCTAATGA ---------- ----------   468
```

```
       TTGCAGAGAC AGGACCTGAC TATTGAAGTT GCAGATTCAT TTTTCTTTCT 513
       | |||||||    ||||     ||   || |  || |||| ||| ||||||
 5     --GGAGAGAC CATCCCTGGG CATCTCAGCT GTGGACTCAT TTTCCTTTCT 516


       GATGTCAAAA ATGTCTTGGG -TAGGCGGGA AGGAGGGTTA GGGAGG-GGT 561.
       |  |||  |   |  || |||  |||| ||  |||||||||   |||||
 10    CACATCAGAC TTTGCTGGGG AGAGGCAGGG AGGAGGGTTG AGGAGGAAGG 566


       AAAATTCCTT AGCTTAGACC TCAGCCTGTG CTGCCCGTCT TCAGCCTAGC 611
       | || |||  ||||| | || |||||||||  ||||| | ||    |  |
 15    GAGATGCCTC AGCTTTGGCC TCAGCCTGCA CTGCCTGCCT AGTGCTCAG- 615


       CGACCTCAGC CTTCCCCTTG CCCAGGGCTC AGCCTGGTGG GCCTCCTCTG 661
                                  || ||| |||||||
 20    ---------- ---------- ----GGTCTC AGCCTGG--- ---------- 628




 25


       TCCAGGGCCC TGAGCTCGGT GGACCCAGGG ATGACATGTC CCTACACCCC 711
       ||   |||    |  | |    ||||       |  | ||  | ||||
 30    --CAACACCC CCACCCC--- --ACCC---- ----CCACCC CCGCCGCCCC 663


       TCCCCTGCCC TAGAGCACAC TGTAGCATTA CAGTGGGTGC CCCCCTTGCC 761
       |||  |||  || || | || || ||||  |  | ||| || |   ||
 35    ATCCCATCCC TACAGAAAAC TGCAGCAAGA CCGTGAGTCC AGCC------ 707


       AGACATGTGG TGGGACAGGG ACCCACTTCA CACACAGGCA ACTGAGGCAG 811
          |||||             ||  | || |||| |||| |||||||||||
 40    -----TGTGG ---------- -CCTGGTCCA CACA-GGGCA ACTGAGGCAG 740


       ACAGCAGCTC AGGCACACTT CTTCTTGGTC TTATTTATTA ---TTGTGTG 858
       ||||||||    ||||| ||  |||||||| || ||||||||||    ||||||
 45    GCAGCAGCTT GAGCACATTT CTTCTTGATC TTATTTATTA TGGTTGTGTG 790


       TTATTTAAAT GAGTGTGTTT GT-CACCGTT GGGGATTGGG GAAGACTGTG 907
       |||||||||| |||| |||   ||   ||| | |||||   ||
 50    TTATTTAAAT GAGTCTGTCA GTATCCCGGT GGGGACATGG ---------- 830


       GCTGCTGGCA CTTGGAGCCA AGGGTTCAGA GACTCAGGGC CCCAGCACTA 957
                  |||  |||   |            || |||| ||||||| |
       ---------- TTTGCTGCCT ATG------- CCCTGGGGGC TCCAGCATTG 863
```

```
    AAGCAGTGGA CCCCAGGAGT CCCTGGTAAT AAGTACTGTG TACAGAATTC 1007
    |||||||||   |  | || ||  |||||| |||  | |||||||  |||| || ||
 5  AAGCAGTGG- GCTCTGGGGT CCCTGGCAAT -ATTACTGTA TACATAACTC  911


    TGCTACCTCA CTGGGGTCCT GGGGCCTCGG AGCCTCATCC GAGGCAGG-- 1055
    ||||||||||         || |  |||||   |  |||| ||  |||||||
10  TGCTACCTCA --------CT GTAGCCTCCA GGTCTCACCC CAGGCAGGAG  953


    --GTCAGGAG AGGGGCAGAA CAGCCGCTCC TGTCTGCCA- GCCAGCAGCC 1102
     |  ||| |  | || ||||  ||  | ||||  |||||||||  |  ||||| ||
15  ATGGGAGGGG A-GGCCAGAG CA-ACACTCC TGTCTGCCAC GGCAGCAACC 1001


    AGCTCTCAGC CAACGAGTAA TTTATTGTTT T-CCTCGTA TTTAAA-TAT 1150
    ||| |||||| ||   | |||  ||||||||||  | | ||  ||  |||||| |||
20  AGCCCTCAGC CATGAAATAA CTTATTGTTT TGTTCTTATA TTTAAAGTAT 1051


    TAAATATGTT AGCAAAGAGT ---TAATATA TAGAAGGGT- ACCTTGAACA 1196
    ||||||  ||  ||||||||||      |||||||  | ||||  |   |||   |||
25  TAAATAGCTT AGCAAAGAGT TAATAATATA TGGAAGAATG GGCTGTTACA 1101


    CT-------- ---------- ---GGGGGAG GG-------- --GACATTGA 1215
    ||                    |||||||  ||          |  || |||
30  CTCAAGGTGA TGTGTAGTGA ATGGGGGGAG GGTGGTGGGT TTGTCACTGA 1151


    ACAAGTTGTT TCATTGACTA TCAAACT-GA AGCCAGAAAT AAAGTTGGTG 1264
    ||||  | || ||||||||||  |||||||| ||  | || |||||  |||| |||||
35  ACAAACT-TT TCATTGACTG TCAAACTAGA AACCGGAAAT AAAGATGGTG 1200


    ACAGATAAAA AA      1276
    |||||||||| ||
40  ACAGATAAAA AA      1212
```

Table 3: Comparison of Human IL-13 and Mouse P600 Amino Acid
Sequences (human above; mouse below). Another form of human
IL-13 has a GLN between amino acids numbered 97 and 98 (position
indicated by |), caused by alternative mRNA splicing.

```
    MET ALA LEU LEU LEU THR THR VAL ILE ALA LEU THR CYS LEU GLY  15
     *   *   *           *       *       *   *       *   *   *
    MET ALA LEU TRP VAL THR ALA VAL LEU ALA LEU ALA CYS LEU GLY  15


    GLY PHE ALA SER PRO GLY PRO VAL PRO PRO SER ─── ─── ─── ───  26
     *       *       *   *   *   *   *       *
    GLY LEU ALA ALA PRO GLY PRO VAL PRO ARG SER VAL SER LEU PRO  30


    THR ALA LEU ARG GLU LEU ILE GLU GLU LEU VAL ASN ILE THR GLN  41
         *       *   *   *   *   *   *       *   *   *   *
    LEU THR LEU LYS GLU LEU ILE GLU GLU LEU SER ASN ILE THR GLN  45


    ASN GLN LYS ALA PRO LEU CYS ASN GLY SER MET VAL TRP SER ILE  56
     *   *           *   *   *   *   *   *   *   *   *   *
    ASP GLN THR ─── PRO LEU CYS ASN GLY SER MET VAL TRP SER VAL  59


    ASN LEU THR ALA GLY MET TYR CYS ALA ALA LEU GLU SER LEU ILE  71
         *       *   *           *       *   *       *   *
    ASP LEU ALA ALA GLY GLY PHE CYS VAL ALA LEU ASP SER LEU THR  74


    ASN VAL SER GLY CYS SER ALA ILE GLU LYS THR GLN ARG MET LEU  86
     *       *       *       *   *           *   *   *       *
    ASN ILE SER ASN CYS ASN ALA ILE TYR ARG THR GLN ARG ILE LEU  89


    SER GLY PHE CYS PRO HIS LYS VAL SER ALA GLY|PHE SER SER LEU 101
         *       *           *                   *   *   *
    HIS GLY LEU CYS ASN ARG LYS ALA PRO THR THR VAL SER SER LEU 104


    HIS VAL ARG ASP THR LYS ILE GLU VAL ALA GLN PHE VAL LYS ASP 116
                 *   *   *   *   *   *   *       *
    PRO ─── ─── ASP THR LYS ILE GLU VAL ALA HIS PHE ILE THR LYS 117


    LEU LEU LEU HIS LEU LYS LYS LEU PHE ARG GLU GLY ARG PHE ASN 131
     *   *           *       *   *   *       *       *
    LEU LEU SER TYR THR LYS GLN LEU PHE ARG HIS GLY PRO PHE ─── 131
```

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The amino acid sequence of the other form of human IL-13 mentioned in the legend to Table 3 is defined in the Sequence Listing by SEQ ID NO: 2.

As used herein, the term "IL-13" describes a protein
5   comprising a protein or peptide segment having the amino acid sequence shown in Table 1, or a fragment thereof. It also refers to a polypeptide which functionally affects cells or subcellular components in a manner similar to the IL-13 allele whose sequence is provided. It also encompasses allelic and other variants, e.g.,
10   metabolic, of the protein described. Typically, it will bind to its corresponding biological receptor with high affinity, e.g., at least about 100 nM, usually better than about 30 nM, preferably better than about 10 nM, and more preferably at better than about 3 nM. The term shall also be used herein to refer to related naturally
15   occurring forms, e.g., allelic and metabolic variants of the human protein.

     This invention also encompasses proteins or peptides having substantial amino acid sequence homology with the amino acid sequence in Table 1, but excluding any protein or peptide which
20   exhibits substantially the same or lesser amino acid sequence homology than does the corresponding P600 protein found in the mouse.

     A polypeptide "fragment", or "segment", is a stretch of amino acid residues of at least about 8 amino acids, generally at least 10
25   amino acids, more generally at least 12 amino acids, often at least 14 amino acids, more often at least 16 amino acids, typically at least 18 amino acids, more typically at least 20 amino acids, usually at least 22 amino acids, more usually at least 24 amino acids, preferably at least 26 amino acids, more preferably at least 28 amino acids, and,
30   in particularly preferred embodiments, at least about 30 or more amino acids. Sequences of segments of different proteins can be compared to one another over appropriate length stretches.

     Amino acid sequence homology, or sequence identity, is determined by optimizing residue matches, if necessary, by
35   introducing gaps as required. See, e.g., Needleham et al., J. Mol. Biol.

48:443 (1970); Sankoff et al., chapter one in *Time Warps, String Edits, and Macromolecules: The Theory and Practice of Sequence Comparsion*, 1983, Addison-Wesley, Reading, MA; and software packages from IntelliGenetics, Mountain View, CA; and the
5   University of Wisconsin Genetics Computer Group, Madison, WI. This changes when considering conservative substitutions as matches.

Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine,
10   threonine; lysine, arginine; and phenylalanine, tyrosine. Homologous amino acid sequences are intended to include natural allelic variations in the provided sequence. Typical homologous proteins or peptides will have from 50-100% homology (if gaps can be introduced), to 60-100% homology (if conservative substitutions are
15   included) with an amino acid sequence segment of Table 1.

Homology will be at least about 50%, generally at least 58%, more generally at least 63%, often at least 69%, more often at least 75%, typically at least 81%, more typically at least 86%, usually at least 90%, more usually at least 93%, preferably at least 95%, and
20   more preferably at least 97%, and in particularly preferred embodiments, at least 98% or more. The degree of homology will vary with the length of the compared segments. Homologous proteins or peptides, such as the allelic variants, will share most biological activities with the embodiment described in Table 1.
25   As used herein, the term "biological activity" is used to describe, without limitation, inducing characteristic cell stimulation, Ig production, cellular differentiation, or cell viability functions, or more structural properties as receptor binding and cross-reactivity with antibodies raised against the same or an allelic variant of the
30   described human IL-13.

The terms ligand, agonist, antagonist, and analog include molecules that modulate the characteristic cellular responses to IL-13 or IL-13-like proteins, as well as molecules possessing the more standard structural binding competition features of ligand-
35   receptor interactions, e.g., where the receptor is a natural receptor or an antibody. The cellular responses likely are mediated through

binding of IL-13 to cellular receptors.   Also, a ligand is a molecule which serves either as a natural ligand to which said receptor, or an analogue thereof, binds, or a molecule which is a functional analogue of the natural ligand.

5   The functional analogue may be a ligand with structural modifications, or may be a wholly unrelated molecule which has a molecular shape which interacts with the appropriate ligand binding determinants.   The ligands may serve as agonists or antagonists, see, e.g., Goodman *et al.*, Eds., *The Pharmacological Bases of Therapeutics*, 10   1990, Pergamon Press, New York.

II. Activities

· The human IL-13 protein has a number of different biological activities.   The human IL-13 is homologous to the mouse P600 protein, but has structural differences.   For example, the human 15   IL-13 gene coding sequence has only about 50% homology with the nucleotide coding sequence of mouse P600.   At the amino acid level, there is about 66% identity.

         The mouse P600 molecule had rather minimally defined biological activities.   In particular, it has the ability to stimulate 20   undifferentiated mouse bone marrow cells to undergo early stages of differentiation.   The mouse P600 protein appears to activate both mouse cells and human cells in this assay.

         The present disclosure also describes new activities which have been discovered using the mouse P600 molecule.   The 25   difference in structure between the human IL-13 and the homologous mouse P600 protein introduce some uncertainty about whether the two proteins will have identical functional properties. However, the handful of identified activities appear to be shared between the homologues.   It is likely that many of the activities of 30   mouse P600 on mouse cells or human cells will also apply to the human IL-13.   In fact, the cross species activities indicate that many structural features are not critical in the function of the molecules.

         In particular, the human IL-13 exhibits a number of identified activities when provided to human cells.   The Examples section

below describes procedures used to study the effects of human
IL-13 on cell viability, morphology, proliferation, and
differentiation. In particular, human IL-13 affects B cells, PBMC,
and macrophages. On B cells, the cytokine affects proliferation,

5   alone or in combination with other cytokines; sustains cell viability;
affects survival; causes modification of Ig surface markers; has
effects specifically on CD40; and affects IgE switching.

On PBMC or macrophages, it induces morphological changes,
causes changes of cell surface markers, affects nitric oxide

10 production, affects IL-1α and IL-6 expression, and affects antibody
dependent cell-mediated cytotoxicity (ADCC). Importantly, the
similarity between IL-4 and IL-13 leads to antagonists of IL-13
whose structures are based upon similar antagonists of IL-4. These
activities can be useful in treating immunological conditions

15 characterized by corresponding disfunctions. See, e.g., *Merck
Manual*, or Paul, *Fundamental Immunology*.

A. B cells

1. Cofactor/factor Proliferation: Cell Viability

Mouse P600 made from *E. coli* has the ability to stimulate or

20 costimulate proliferation of large *in vivo* activated mouse B cells.
The combination of IL-4, soluble anti-CD40, and mouse P600 induces
proliferation of these cells. However, since the large *in vivo*
activated mouse B cells may contain some monocytes and other cells,
other cells may be induced to secrete various growth factors which

25 support the proliferation observed. Thus, the mouse P600
stimulates the B cells, either directly or indirectly, alone or in
combination with other factors. Human IL-13 should exhibit similar
biological activity.

2. Sustained Survival of B Cells: Selectivity

30 IL-13 enhanced the DNA synthesis of B cells activated through
their antigen receptor. This induction was dose dependent, and was
comparable in effect to IL-10, but less than IL-2 or IL-4. The time

course of the effect was also different from IL-2 and IL-4.
Similarly, B cells activated through their CD40 receptor were also
affected, again in a dose dependent manner, and comparable to IL-4
and IL-10 effects. The kinetics of the effects exhibited a different
5   time course from IL-4 or IL-10 responses. The combination of
IL-10 and IL-13 exhibited additive effects, but IL-13 did not appear
to increase any IL-4 effects. This suggested, with other data, that
these two cytokines may share some components in signal
transduction, though other data show some independence of effects.
10   More specifically, IL-13 induced expression of various Ig's,
particularly IgE. The target cell population for IL-13 also appears
more restricted than for IL-4. Thus, although IL-4 and IL-13 share
many biological properties, their signal transduction pathways are
physiologially and mechanistically distinguishable.

15   3. <u>Modification of Ig Production</u>

B cells activated by B21 T cell clone, their membranes, or anti-
CD40 appear to exhibit modified Ig production patterns after
exposure to mouse P600 or human IL-13. The levels of production
of various Ig molecule subtypes when human IL-13 is co-
20   administered to B cells with an inducing agent, e.g., activated B21 T
cells, membranes from activated B21 T cells, or anti-CD40 antibodies
were increased, particularly IgE. The changes in Ig production are
suggestive of accelerated differentiation including IgG4 and/or IgE
class switching. Both possibilities are consistent with a
25   differentiation effect caused by mouse P600 or human IL-13.
A similar induction of IgE and IgG4 synthesis by IL-13 was
observed when B cells were stimulated with CD40-L, as described
below.

         4. <u>Effects on CD40-mediated B Cell Proliferation and</u>
30   <u>Differentiation</u>

The effects of anti-CD40 antibody or CD40 ligand on B cell
proliferation were enhanced in the presence of either IL-4 or IL-13.
Both cytokin s had comparable effects in spite of their significant

sequence divergence. The B cell proliferation was accompanied by
induction of IgM, IgG4, total IgG, and IgE levels. IgA was not
stimulated. The two cytokines appeared to act through different
structural mechanisms as anti-IL-4 antibodies blocked IL-4 effects,
5   but not IL-13 effects. The effects on IgE suggest that IL-13
contributes to IgE production and is an important factor in
controlling IgE mediated allergic reactions.

5. IgE Switching

IL-13 induced IgG4 and IgE synthesis by unfractionated
10   peripheral blood mononuclear cells (PBMNC) and highly purified B
cells cultured in the presence of activated $CD4^+$ T cells or their
membranes. IL-13-induced IgG4 and IgE synthesis was IL-4-
independent, since it was not affected by neutralizing anti-IL-4
monoclonal antibody (mAb). Highly purified $sIgD^+$ B cells could also
15   be induced to produce IgG4 and IgE by IL-13, indicating that the
production of these isotypes reflected IgG4 and IgE switching and
not a selective outgrowth of committed B cells.
        IL-4 and IL-13, added together at optimal concentrations, had
no additive or synergistic effect, suggesting that common signaling
20   pathways may be involved. This notion is supported by the
observation that IL-13, like IL-4, induced CD23 expression on B cells
and enhanced CD72, surface IgM (sIgM), and class II MHC antigen
expression. In addition, like IL-4, IL-13 induced germline ε
transcription in highly purified B cells. Collectively, these data
25   indicated that IL-13 is another T cell-derived cytokine that, in
addition to IL-4, efficiently directs naive human B cells to switch to
IgG4 and IgE production.

B. PBMC and Macrophages

1. Induction of Morphological Change

30   The mouse P600 also induced morphological changes in
adherent human peripheral blood mononuclear cells. The treated
cells exhibited significantly different morphology and clusters of

small cells. The generic cells rounded up, and there was evidence of clonal proliferation, observations which were consistent with induction of cell proliferation.

2. Modification of Cell Surface Markers

5   Mouse P600 induced significant changes in the cell surface markers of adherent cells from peripheral blood. These adherent cells were mostly monocytes, e.g., macrophage precursors, but also included more differentiated cell types, dendritic cells, and some B cells.

10   Many of the cell surface makers on these adherent cells were up regulated or down regulated, or their dispersion in expression level changed. The following markers tended to increase on a per cell basis: CD11b, CD11c, class II MHC (as measured by binding of monoclonal antibodies Q5/13 or PdV5.2), CD 23, and CD18. In

15   contrast, per cell expression of the following decreased: CD32, CD16, IL-2Rα, and CD14. The homogeneity of per cell expression changed for CD32, and CD14. There was no change for CD11α, CD54, and CD58. Although in one case there was no change for CD44 and class I MHC, other experiments indicated increases in expression levels.

20   These changes in expression level were detectable also at 10 days, and in certain cases exhibited a more dramatic shift, whereas others showed a lesser shift. Depending on subsets of cells, some features may have been lost by ten days.

In spite of the sequence divergence of the mouse P600 and the

25   human IL-13, the two molecules seemed to cause similar changes in the adherent human cells. It is likely that activities found for one of the molecules will be found also by the other. In addition, the molecules appeared to exhibit cross-species activities, e.g., the mouse P600 was active on human cells, and the human IL-13 was

30   active on mouse cells.

3. Nitric Oxide Synthesis

IL-13 (P600) was assayed by its LPS stimulated inhibitiory effect on the production of nitric oxide (NO) by GM-CSF-derived bone marrow macrophages. IFN-γ induced NO production, while
5   IL-4 or IL-13 inhibited NO production.

4. Effect on IL-1α, IL-6, IL-10 and TNF-α Production

IL-4 and IL-13 inhibited the production of IL-1α, IL-6, IL-10, and TNF-α by LPS-activated human monocytes. The inhibitory effects of IL-4 and IL-13 on cytokine production by LPS activated
10   human monocytes were independent of IL-10, since IL-4 and IL-13 inhibited the production of IL-1α, IL-6, and TNF-α in the presence of neutralizing anti-IL-10 mAb 19F1.

5. Antibody-dependent Cell-mediated Cytotoxicity

IL-13 induced significant changes in the phenotype of
15   monocytes. Like IL-4, it enhanced the expression of CD11b, CD11c, CD18, CD29, CD49e (VLA-5), class II MHC, CD13, and CD23 whereas it decreased the expression of CD64, CD32, CD16, and CD14 in a dose dependent manner. IL-13 induced upregulation of class II MHC antigens and its downregulatory effects on CD64, CD32, and CD16
20   expression were prevented by IL-10.
IFN-γ could also partially prevent the IL-13 induced downregulation of CD64, but not that of CD32 and CD16. However, IL-13 strongly inhibited spontaneous and IL-10 or IFN-γ induced antibody dependent cell-mediated cytotoxicity (ADCC) activity of
25   human monocytes toward anti-IgD coated Rh+ erythrocytes, indicating that the cytotoxic activity of monocytes was inhibited. These results indicated that IL-13 has anti-inflammatory and immunoregulatory activities.

30

C. IL-4 Antagonist: Interactions

Observations that the hIL-4.Y124D antagonist competitively inhibited the biological action on TF-1 cells of both hIL-4 and IL-13 demonstrated a relationship between IL-4R and IL-13R. The ability
5   of mIL-13 to compete for I^{125}-hIL-4 binding to TF-1 cells confirmed the commonalty of IL-4R and IL-13R. This relatedness may also have been expected from the similar biological responses known to be elicited by hIL-4 and IL-13, and perhaps from the close linkage of the IL-4 and IL-13 genes in both humans and mice.
10 See, e.g., Morgan *et al.*, *Nucleic Acids Res.* 20:5173 (1992), and other experiments herein. A straightforward explanation of the above observations would be that IL-4 and IL-13 act through the same receptor.

D. Biological Relevance

15   The mouse P600 protein can sustain or promote the proliferation of large *in vivo* activated B cells. As such, the factor appears to be either a stimulatory or costimulatory factor useful in promoting activated B cell growth. Human   IL-13 is therefore expected to be a useful factor in circumstances where activated B
20 cell growth is desired.

These include genetic, developmental, or acquired immune system deficiencies, e.g., congenital aglobulinemias, immature infants, or chemotherapy patients. *In vitro* experiments would be performed to determine what effects IL-13 possesses. In particular,
25 dose response relationships for various immunological assays will be tested with the compositions of this invention. See, e.g., Coligan *et al.*, *Current Protocols in Immunology*, 1991, Greene/Wiley, New York.

Regarding the proliferative response, mouse P600 induces changes in morphology of the monocyte cells. The monocyte cells
30 consist primarily of macrophage precursors, and similar results should apply to monocyte equivalents found in organs or tissues other than the peripheral blood, e.g., the aveolar, intraperitoneal, or spleen/lymph macrophage precursors. The IL-13 or antagonist, e.g., antibody or IL-4 antagonist, would be indicated for conditions

where regulation of localized or systemic immune responses is
desired and appropriate. The effects on class II MHC are especially
relevant in these contexts.

Besides a growth factor/cofactor activity, human IL-13 also
5   affects differentiation of various cells of the immune system. For
instance, in activated B cells, it accelerates or promotes the
differentiation of Ig producing cells. It induces the B cells to
produce Ig molecules characteristic of later or faster differentiation.
As such, the human IL-13 and mouse P600 appear to be a
10   differentiation factor for B cells.

Thus, Ig production should be regulatable by IL-13, alone or in
combination with other factors. Agonists and antagonists, when
provided in appropriate amounts and schedules, will be useful in
treating or controlling abnormal B cell conditions, or to accelerate or
15   decelerate B cell differentiation when appropriate.

Peripheral blood monocytes are also sensitive to the presence
of both human IL-13 and mouse P600. These cells, consisting
primarily of macrophage precursors and more differentiated cell
types, exhibit both a proliferative response and a differentiation
20   response.

In one context, IL-4 is appropriate in antitumor situations, e.g.,
to stimulate an endogenous response to counter the tumor; IL-13
should also be a useful therapeutic. In a different context of
proliferative disorder, after radiotherapy or chemotherapy, where
25   the immune funtion is typically compromised, IL-13 would be
useful to restore function by promoting recovery and differentiation
of the remaining immune function. See, e.g., Moller (ed), "Fc
Receptors" in *Immunological Reviews 125*:1 (1992). Similar
problems exist in transplantation contexts, as well as in other
30   genetic or developmental immunodeficiencies, e.g., in newborn
infants. See, e.g., Baker *et al., N. Eng. J. Med. 327*:213 (1992).

In fact, the role of IL-13 in promoting restoration of immune
function under these circumstances is supported by the cell marker
changes observed. With respect to cell marker differentiation, the
35   general trend is that the class II MHC markers are affected. Also,
CD23 is affected. The effects on class II MHC markers indicate that

systemic responsiveness to infections can be modulated with IL-13 or mouse P600, or agonists or antagonists thereof.

The observed decreases in CD32 and CD16 indicated a lowered receptor for IgG Fc, which would be correlated with a lessened response to infections. If so, an IL-13 antagonist, or mouse P600 antagonist, would be useful in stimulating an immunoglobulin-mediated response. This antagonistic activity could lead to increased Fcγ receptor expression and functional increase in opsonization and clearance of infective particles.

Antagonists to IL-13, e.g., antibodies or IL-4 antagonist, would be indicated for modulating B cell growth and proliferation, perhaps reflecting excessive humoral responses. Various autoimmune conditions or hyperimmunoglobulinemias should respond to treatment with appropriate amounts of antagonists administered over defined schedules. IL-4 antagonist will be a preferred antagonist for IL-13 effects.

IL-13 mediates changes in CD11 marker expression, which are associated with cell adhesion, e.g., cell-cell contacts, Thus, increasing CD11 should facilitate cellular interaction and the functional results therefrom. See also Springer *et al., Leukocyte Adhesion Molecules,* 1988, Springer-Verlag, New York.

Like IL-4, IL-13 induces IgG4 and IgE switching and IgG4 and IgE synthesis. IgE antibodies are major mediators of allergic reactions. Allergen-specific antibodies of the IgE isotype have the specific ability to bind to high affinity Fc receptors for IgE (FcεRI) on mast cells and basophils. Binding of the relevant allergen to these receptor-bound IgE antibodies results in cross-linking of the receptor and activation of the mast cells and basophils. This results in degranulation of these cells and the release of mediators of allergic reactions such as histamine, prostaglandins and proteases, which cause immediate-type hypersensitivity reactions in the various target organs, e.g., nose airways, lungs, gut and skin.

In addition, IL-13 like IL-4 induces the expression of the low affinity receptor for IgE (FcεRII, or CD23) on B cells and monocytes, and the subsequent release of a soluble form of CD23. Soluble CD23 enhances the production of IgE [see, e.g., Pene *et al., Eur. J. Immunol.*

18:929 (1988); Aubry *et al.*, *Nature 358*:505 (1992)]. Therefore,
downregulation of IgE synthesis and soluble CD23 production reduce
or inhibit IgE-mediated allergic diseases. IL-4 and/or IL-13
antagonists such as antibodies, or IL-4 mutant proteins like Y124D

5   or similar mutant IL-13 proteins that compete for IL-4/IL-13
receptor binding, would be useful for blocking IgE production.

III.   Nucleic Acids

This invention contemplates use of isolated nucleic acid or
fragments which encode this or a closely related protein, or

10   fragments thereof, to encode a biologically active corresponding
polypeptide. In addition, this invention covers isolated or
recombinant DNA which encodes a biologically active protein or
polypeptide having characteristic IL-13 activity. Typically, the
nucleic acid is capable of hybridizing, under appropriate conditions,

15   with a nucleic acid sequence segment shown in Table 1.
Said biologically active protein or polypeptide can be a full
length protein, or fragment, and will typically have a segment of
amino acid sequence highly homologous to one shown in Table 1.
Further, this invention covers the use of isolated or recombinant

20   nucleic acid, or fragments thereof, which encode proteins having
fragments which are homologous to the disclosed IL-13 protein. The
isolated nucleic acids can have the respective regulatory sequences
in the 5' and 3' flanks, e.g., promoters, enhancers, poly-A addition
signals, and others from the natural gene.

25   An "isolated" nucleic acid is a nucleic acid, e.g., an RNA, DNA, or
a mixed polymer, which is substantially pure, e.g., separated from
other components which naturally accompany a native sequence,
such as ribosomes, polymerases, and flanking genomic sequences
from the originating species. The term embraces a nucleic acid

30   sequence which has been removed from its naturally occurring
environment, and includes recombinant or cloned DNA isolates,
which are thereby distinguishable from naturally occurring
compositions, and chemically synthesized analogues or analogues
biologically synthesized by heterologous systems. A substantially

pure molecule includes isolated forms of the molecule, either completely or substantially pure.

An isolated nucleic acid will generally be a homogeneous composition of molecules, but will, in some embodiments, contain heterogeneity, preferably minor. This heterogeneity is typically found at the polymer ends or portions not critical to a desired biological function or activity.

A "recombinant" nucleic acid is defined either by its method of production or its structure. In reference to its method of production, e.g., a product made by a process, the process is use of recombinant nucleic acid techniques, e.g., involving human intervention in the nucleotide sequence. Typically this intervention involves *in vitro* manipulation, although under certain circumstances it may involve more classical animal breeding techniques.

Alternatively, it can be a nucleic acid made by generating a sequence comprising fusion of two fragments which are not naturally contiguous to each other, but is meant to—exclude products of nature, e.g., naturally occurring mutants as found in their natural state. Thus, for example, products made by transforming cells with any unnaturally occurring vector is encompassed, as are nucleic acids comprising sequence derived using any synthetic oligonucleotide process. Such a process is often done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a restriction enzyme sequence recognition site.

In still another alternative, the process is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the commonly available natural forms, e.g., encoding a fusion protein. Restriction enzyme recognition sites are often the target of such artificial manipulations, but other site specific targets, e.g., promoters, DNA replication sites, regulation sequences, control sequences, or other useful features may be incorporated by design.

A similar concept is intended for a recombinant, e.g., fusion, polypeptide. Specifically included are synthetic nucleic acids which, by genetic code redundancy, encode similar polypeptides to

fragments of the interleukins, and fusions of sequences from various different interleukin or related molecules, e.g., growth factors.

A "fragment" in a nucleic acid context is a contiguous segment of at least about 17 nucleotides, generally at least 20 nucleotides,

5   more generally at least 23 nucleotides, ordinarily at least 26 nucleotides, more ordinarily at least 29 nucleotides, often at least 32 nucleotides, more often at least 35 nucleotides, typically at least 38 nucleotides, more typically at least 41 nucleotides, usually at least 44 nucleotides, more usually at least 47 nucleotides, preferably at

10 least 50 nucleotides, more preferably at least 53 nucleotides, and in particularly preferred embodiments will be at least 56 or more nucleotides. Typically, fragments of different genetic sequences can be compared to one another over appropriate length stretches.

A nucleic acid which codes for an IL-13 will be particularly

15 useful to identify genes, mRNA, and cDNA species which code for the IL-13 or closely related proteins, as well as DNAs which code for allelic or other genetic variants, e.g., from different individuals. Preferred probes for such screens are those regions of the interleukin which are conserved between different allelic variants,

20 and will preferably be full length or nearly so. In other situations, allele specific sequences will be more useful.

This invention further covers recombinant nucleic acid molecules and fragments having a nucleic acid sequence identical to or highly homologous to the isolated DNA set forth herein. In

25 particular, the sequences will often be operably linked to DNA segments which control transcription, translation, and DNA replication. These additional segments typically assist in expression of the desired nucleic acid segment.

Homologous nucleic acid sequences, when compared to one

30 another or Table 1 sequences, exhibit significant similarity. The standards for homology in nucleic acids are either measures for homology generally used in the art by sequence comparison or based upon hybridization conditions. Comparative hybridization conditions are described in greater detail below.

35   "Substantial homology" in the nucleic acid sequence comparison context means either that the segments, or their

complementary strands, when compared, are identical when
optimally aligned, with appropriate nucleotide insertions or
deletions, in at least about 60% of the nucleotides, generally at least
66%, ordinarily at least 71%, often at least 76%, more often at least
5   80%, usually at least 84%, more usually at least 88%, typically at
least 91%, more typically at least about 93%, preferably at least
about 95%, more preferably at least about 96 to 98% or more, and in
particular embodiments, as high at about 99% or more of the
nucleotides.

10   Alternatively, substantial homology exists when the segments
will hybridize under selective hybridization conditions, to a strand
or its complement, typically using a sequence derived from Table 1.
Typically, selective hybridization will occur when there is at least
about 55% homology over a stretch of at least about 14 nucleotides,
15   more typically at least about 65%, preferably at least about 75%, and
more preferably at least about 90%. See, Kanehisa, *Nucleic Acids
Res. 12*:203 (1984).

The length of homology comparison, as described, may be over
longer stretches, and in certain embodiments will be over a stretch
20   of at least about 17 nucleotides, generally at least about 20
nucleotides, ordinarily at least about 24 nucleotides, usually at least
about 28 nucleotides, typically at least about 32 nucleotides, more
typically at least about 40 nucleotides, preferably at least about 50
nucleotides, and more preferably at least about 75 to 100 or more
25   nucleotides.

Stringent conditions, in referring to homology in the
hybridization context, will be stringent combined conditions of salt,
temperature, organic solvents, and other parameters typically
controlled in hybridization reactions.   Stringent temperature
30   conditions will usually include temperatures in excess of about 30°C,
more usually in excess of about 37°C, typically in excess of about
45°C, more typically in excess of about 55°C, preferably in excess of
about 65°C, and more preferably in excess of about 70°C.   Stringent
salt conditions will ordinarily be less than about 1000 mM, usually
35   less than about 500 mM, more usually less than about 400 mM,
typically less than about 300 mM, preferably less than about 200

mM, and more preferably less than about 150 mM. However, the combination of parameters is much more important than the measure of any single parameter. See, e.g., Wetmur *et al., J. Mol. Biol. 31*:349 (1968).

5   The isolated DNA can be readily modified by nucleotide substitutions, nucleotide deletions, nucleotide insertions, and inversions of nucleotide stretches. These modifications result in novel DNA sequences which encode this protein, its derivatives, or proteins having IL-13 activity. These modified sequences can be
10   used to produce mutant proteins (muteins) or to enhance the expression of variant species. Enhanced expression may involve gene amplification, increased transcription, increased translation, and other mechanisms. Such mutant IL-13 derivatives include predetermined or site-specific mutations of the protein or its
15   fragments.

         "Mutant IL-13" as used herein encompasses a polypeptide otherwise falling within the homology definition of the human IL-13 as set forth above, but having an amino acid sequence which differs from that of human IL-13 as found in nature, whether by way of
20   deletion, substitution, or insertion. In particular, "site specific mutant IL-13" encompasses a protein having substantial homology with a protein of Table 1, and typically shares most of the biological activities of the form disclosed herein.

         Although site specific mutation sites are predetermined,
25   mutants need not be site specific. Human IL-13 mutagenesis can be achieved by making amino acid insertions or deletions in the gene, coupled with expression. Substitutions, deletions, insertions, or any combinations may be generated to arrive at a final construct. Insertions include amino- or carboxy- terminal fusions. Random
30   mutagenesis can be conducted at a target codon and the expressed human IL-13 mutants can then be screened for the desired activity. Methods for making substitution mutations at predetermined sites in DNA having a known sequence are well known in the art, e.g., by M13 primer mutagenesis. See also Sambrook *et al.* (1989) and
35   Ausubel *et al.* (1987 and periodic Supplements).

The mutations in the DNA normally should not place coding sequences out of reading frames and preferably will not create complementary regions that could hybridize to produce secondary mRNA structure such as loops or hairpins.

5   The phosphoramidite method described by Beaucage *et al.*, *Tetra. Letts.* 22:1859 (1981), will produce suitable synthetic DNA fragments. A double stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the

10   complementary strand using DNA polymerase with an appropriate primer sequence.

Polymerase chain reaction (PCR) techniques can often be applied in mutagenesis. Alternatively, mutagenisis primers are commonly used methods for generating defined mutations at

15   predetermined sites.

IV.   Proteins. Peptides

As described above, the present invention encompasses the human IL-13 whose sequence is disclosed in Table I and described above. Allelic and other variants are also contemplated.

20   "Substantially pure", in the polypeptide context, typically means that the protein is free from other contaminating proteins, nucleic acids, and other biologicals derived from the original source organism. Purity may be assayed by standard methods, and will ordinarily be at least about 40% pure, more ordinarily at least about

25   50% pure, generally at least about 60% pure, more generally at least about 70% pure, often at least about 75% pure, more often at least about 80% pure, typically at least about 85% pure, more typically at least about 90% pure, preferably at least about 95% pure, more preferably at least about 98% pure, and in most preferred

30   embodiments, at least 99% pure. The analysis may be weight or molar percentages, evaluated, e.g., by gel staining, spectrophotometry, or terminus labelling.

The present invention also provides recombinant proteins, e.g., heterologous fusion proteins using segments from this human

protein. A heterologous fusion protein is a fusion of proteins or segments which are naturally not normally fused in the same manner. Thus, the fusion product of a growth factor with an interleukin is a continuous protein molecule having sequences fused in a typical peptide linkage, typically made as a single translation product and exhibiting properties derived from each source peptide. A similar concept applies to heterologous nucleic acid sequences.

In addition, new constructs may be made from combining similar functional or structural domains from other related proteins, e.g., growth factors or other cytokines. For example, receptor-binding or other segments may be "swapped" between different new fusion polypeptides or fragments. See, e.g., Cunningham *et al.*, *Science 243*:1330 (1989); and O'Dowd *et al., J. Biol. Chem. 263*:15985 (1988).

Thus, new chimeric polypeptides exhibiting new combinations of specificities will result from the functional linkage of receptor-binding specificities. For example, the receptor binding domains from other related ligand molecules may be added or substituted for other domains of this or related proteins. The resulting protein will often have hybrid function and properties. For example, a fusion protein may include a targetting domain which may serve to provide sequestering of the fusion protein to a particular organ, e.g., a ligand portions which is specifically bound by spleen cells and would serve to accumulate in the spleen.

Candidate fusion partners and sequences can be selected from various sequence data bases, e.g., GenBank, c/o IntelliGenetics, Mountain View, CA; and BCG, University of Wisconsin Biotechnology Computing Group, Madison, WI.

"Derivatives" of the human IL-13 include amino acid sequence mutants, glycosylation variants, metabolic derivatives and covalent or aggregative conjugates with other chemical moieties. Covalent derivatives can be prepared by linkage of functionalities to groups which are found in the IL-13 amino acid side chains or at the N- or C- termini, e.g., by means which are well known in the art. These derivatives can include, without limitation, aliphatic esters or amides of the carboxyl terminus, or of residues containing carboxyl

side chains, O-acyl derivatives of hydroxyl group-containing
residues, and N-acyl derivatives of the amino terminal amino acid or
amino-group containing residues, e.g., lysine or arginine.   Acyl
groups are selected from the group of alkyl-moieties including C3 to
5   C18 normal alkyl, thereby forming alkanoyl aroyl species.

In particular, glycosylation alterations are included, e.g., made
by modifying the glycosylation patterns of a polypeptide during its
synthesis and processing, or in further processing steps.   Particularly
preferred means for accomplishing this are by exposing the
10   polypeptide to glycosylating enzymes derived from cells which
normally provide such processing, e.g., mammalian glycosylation
enzymes.   Deglycosylation enzymes are also contemplated. Also
embraced are versions of the same primary amino acid sequence
which have other minor modifications, including phosphorylated
15   amino acid residues, e.g., phosphotyrosine, phosphoserine, or
phosphothreonine.

A major group of derivatives are covalent conjugates of the
interleukin or fragments thereof with other proteins of
polypeptides.   These derivatives can be synthesized in recombinant
20   culture such as N- or C-terminal fusions or by the use of agents
known in the art for their usefulness in cross-linking proteins
through reactive side groups. Preferred derivatization sites with
cross-linking agents are at free amino groups, carbohydrate
moieties, and cysteine residues.

25   Fusion polypeptides between the interleukin and other
homologous or heterologous proteins are also provided.   Homologous
polypeptides may be fusions between different growth factors,
resulting in, for instance, a hybrid protein exhibiting ligand
specificity for multiple different receptors, or a ligand which may
30   have broadened or weakened specificity of binding to its receptor.
Likewise, heterologous fusions may be constructed which would
exhibit a combination of properties or activities of the derivative
proteins.

Typical examples are fusions of a reporter polypeptide, e.g.,
35   luciferase, with a segment or domain of a receptor, e.g., a ligand-
binding segment, so that the pr sence or location of a desired ligand

may be easily determined. See, e.g., Dull *et al.*, U.S. Patent No. 4,859,609. Other gene fusion partners include glutathione-S-transferase (GST), bacterial ß-galactosidase, trpE, Protein A, ß-lactamase, alpha amylase, alcohol dehydrogenase, and yeast alpha
5   mating factor. See, e.g., Godowski *et al.*, *Science 241*:812 (1988).

The phosphoramidite method described by Beaucage *et al.*, *Tetra. Letts.* 22:1859 (1981), will produce suitable synthetic DNA fragments. A double-stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the
10   strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

. Such polypeptides may also have amino acid residues which have been chemically modified by phosphorylation, sulfonation,
15   biotinylation, or the addition or removal of other moieties, particularly those which have molecular shapes similar to phosphate groups. In some embodiments, the modifications will be useful labelling reagents, or serve as purification targets, e.g., affinity ligands.

20   Fusion proteins will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation and expression are described generally, for example, in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2d ed.), 1989,Vols. 1-3, Cold Spring
25   Harbor Laboratory; and Ausubel *et al.* (eds), *Current Protocols in Molecular Biology*, 1987 and periodic supplements, Greene/Wiley, New York. Techniques for synthesis of polypeptides are described, for example, in Merrifield, *J. Am. Chem. Soc. 85*:2149 (1963); Merrifield, *Science 232*:341 (1986); and Atherton *et al.*, *Solid Phase*
30   *Peptide Synthesis: A Practical Approach*, 1989, IRL Press, Oxford.

This invention also contemplates the use of derivatives of the human IL-13 other than variations in amino acid sequence or glycosylation. Such derivatives may involve covalent or aggregative association with chemical moieties. These derivatives generally fall
35   into three classes: (1) salts, (2) side chain and terminal residue covalent modifications, and (3) adsorption complexes, for example

with cell membranes. Such covalent or aggregative derivatives are useful as immunogens, as reagents in immunoassays, or in purification methods such as for affinity purification of a receptor or other binding molecule, e.g., an antibody.

5   For example, the human IL-13 ligand can be immobilized by covalent bonding to a solid support such as cyanogen bromide-activated Sepharose, by methods which are well known in the art, or adsorbed onto polyolefin surfaces, with or without glutaraldehyde cross-linking, for use in the assay or purification of IL-13 receptor,

10   antibodies, or other similar molecules. The IL-13 can also be labelled with a detectable group, for example radioiodinated by the chloramine T procedure, covalently bound to rare earth chelates, or conjugated to another fluorescent moiety for use in diagnostic assays.

15   The human IL-13 of this invention can be used as an immunogen for the production of antisera or antibodies specific for the interleukin or any fragments thereof. The purified interleukin can be used to screen monoclonal antibodies or antigen-binding fragments prepared by immunization with various forms of impure

20   preparations containing the protein. In particular, the term "antibodies" also encompasses antigen binding fragments of natural antibodies. The purified interleukin can also be used as a reagent to detect any antibodies generated in response to the presence of elevated levels of expression, or immunological disorders which lead

25   to antibody production to the endogenous cytokine.

Additionally, IL-13 fragments may also serve as immunogens to produce the antibodies of the present invention, as described immediately below. For example, this invention contemplates antibodies having binding affinity to or being raised against the

30   amino acid sequence shown in Table 1, fragments thereof, or homologus peptides. In particular, this invention contemplates antibodies having binding affinity to, or having been raised against, specific fragments which are predicted to be, or actually are, exposed at the exterior protein surface of the native cytokine.

35   The blocking of physiological response to these interleukins may result fr m the inhibition of binding of the ligand to the

receptor, likely through competitive inhibition. Thus, *in vitro* assays
of the present invention will often use antibodies or ligand binding
segments of these antibodies, or fragments attached to solid phase
substrates. These assays will also allow for the diagnostic

5   determination of the effects of either binding region mutations and
modifications, or ligand mutations and modifications, e.g., ligand
analogues.

This invention also contemplates the use of competitive drug
screening assays, e.g., where neutralizing antibodies to the

10   interleukin or fragments compete with a test compound for binding
to a receptor or antibody. In this manner, the neutralizing
antibodies or fragments can be used to detect the presence of any
polypeptide which shares one or more binding sites to a receptor
and can also be used to occupy binding sites on a receptor that

15   might otherwise bind an interleukin.

V.   Making Nucleic Acids and Protein

DNA which encodes the protein or fragments thereof can be
obtained by chemical synthesis, screening cDNA libraries, or by
screening genomic libraries prepared from a wide variety of cell

20   lines or tissue samples. Natural sequences can be isolated using
standard methods and the sequences provided herein, e.g., in Table 1.

This DNA can be expressed in a wide variety of host cells for
the synthesis of a full-length human interleukin or fragments which
can in turn, for example, be used to generate polyclonal or

25   monoclonal antibodies; for binding studies; for construction and
expression of modified agonist/antagonist molecules; and for
structure/function studies. Each variant or its fragments can be
expressed in host cells that are transformed or transfected with
appropriate expression vectors. These molecules can be

30   substantially free of protein or cellular contaminants, other than
those derived from the recombinant host, and therefore are
particularly useful in pharmaceutical compositions when combined
with a pharmaceutically acceptable carrier and/or diluent. The

human protein, or portions thereof, may be expressed as fusions with other proteins.

Expression vectors are typically self-replicating DNA or RNA constructs containing the desired receptor gene or its fragments,
5   usually operably linked to suitable genetic control elements that are recognized in a suitable host cell. These control elements are capable of effecting expression within a suitable host. The specific type of control elements necessary to effect expression will depend upon the eventual host cell used.

10   Generally, the genetic control elements can include a prokaryotic promoter system or a eukaryotic promoter expression control system, and typically include a transcriptional promoter, an optional operator to control the onset of transcription, transcription enhancers to elevate the level of mRNA expression, a sequence that
15   encodes a suitable ribosome binding site, and sequences that terminate transcription and translation. Expression vectors also usually contain an origin of replication that allows the vector to replicate independently of the host cell.

The vectors of this invention include those which contain DNA
20   which encodes a protein, as described, or a fragment thereof encoding a biologically active equivalent polypeptide. The DNA can be under the control of a viral promoter and can encode a selection marker. This invention further contemplates use of such expression vectors which are capable of expressing eukaryotic cDNA coding for
25   such a protein in a prokaryotic or eukaryotic host, where the vector is compatible with the host and where the eukaryotic cDNA coding for the receptor is inserted into the vector such that growth of the host containing the vector expresses the cDNA in question.

Usually, expression vectors are designed for stable replication
30   in their host cells or for amplification to greatly increase the total number of copies of the desirable gene per cell. It is not always necessary to require that an expression vector replicate in a host cell, e.g., it is possible to effect transient expression of the interleukin protein or its fragments in various hosts using vectors
35   that do not contain a replication origin that is recognized by the host

cell. It is also possible to use vectors that cause integration of the
human protein or its fragments into the host DNA by recombination.

Vectors, as used herein, comprise plasmids, viruses,
bacteriophage, integratable DNA fragments, and other vehicles
5   which enable the integration of DNA fragments into the genome of
the host. Expression vectors are specialized vectors which contain
genetic control elements that effect expression of operably linked
genes. Plasmids are the most commonly used form of vector but all
other forms of vectors which serve an equivalent function and
10 which are, or become, known in the art are suitable for use herein.
See, e.g., Pouwels *et al., Cloning Vectors: A Laboratory Manual*, 1985
and Supplements, Elsevier, N.Y.; and Rodriquez *et al.* (eds), *Vectors:
A Survey of Molecular Cloning Vectors and Their Uses*, 1988,
Buttersworth, Boston.

15   Transformed cells are cells, preferably mammalian, that have
been transformed or transfected with receptor vectors constructed
using recombinant DNA techniques. Transformed host cells usually
express the desired protein or its fragments, but for purposes of
cloning, amplifying, and manipulating its DNA, do not need to
20 express the subject protein. This invention further contemplates
culturing transformed cells in a nutrient medium, thus permitting
the interleukin to accumulate in the culture. The protein can be
recovered, either from the culture or from the culture medium.

For purposes of this invention, nucleic sequences are operably
25 linked when they are functionally related to each other. For
example, DNA for a presequence or secretory leader is operably
linked to a polypeptide if it is expressed as a preprotein or
participates in directing the polypeptide to the cell membrane or in
secretion of the polypeptide. A promoter is operably linked to a
30 coding sequence if it controls the transcription of the polypeptide; a
ribosome binding site is operably linked to a coding sequence if it is
positioned to permit translation. Usually, operably linked means
contiguous and in reading frame, however, certain genetic elements
such as repressor genes are not contiguously linked but still bind to
35 operator sequences that in turn control expression.

Suitable host cells include prokaryotes, lower eukaryotes, and
higher eukaryotes. Prokaryotes include both gram negative and
gram positive organisms, e.g., *E. coli* and   *B. subtilis*. Lower
eukaryotes include yeasts, e.g., *S. cerevisiae* and *Pichia*, and species

5   of the genus *Dictyostelium*. Higher eukaryotes include established
tissue culture cell lines from animal cells, both of non-mammalian
origin, e.g., insect cells, and birds, and of mammalian origin, e.g.,
human, primates, and rodents.

Prokaryotic host-vector systems include a wide variety of

10   vectors for many different species. As used herein, *E. coli* and its
vectors will be used generically to include equivalent vectors used
in other prokaryotes. A representative vector for amplifying DNA is
pBR322 or many of its derivatives. Vectors that can be used to
express the receptor or its fragments include, but are not limited to,

15   such vectors as those containing the lac promoter (pUC-series); trp
promoter (pBR322-trp); Ipp promoter (the pIN-series); lambda-pP
or pR promoters (pOTS); or hybrid promoters such as ptac (pDR540).
See Brosius *et al.*, "Expression Vectors Employing Lambda-, trp-, lac-,
and Ipp-derived Promoters", in *Vectors: A Survey of Molecular*

20   *Cloning Vectors and Their Uses*, (eds. Rodriguez and Denhardt), 1988,
Buttersworth, Boston, Chapter 10, pp. 205-236.

Lower eukaryotes, e.g., yeasts and *Dictyostelium*, may be
transformed with IL-13 sequence containing vectors. For purposes
of this invention, the most common lower eukaryotic host is the

25   baker's yeast, *Saccharomyces cerevisiae*. It will be used to
generically represent lower eukaryotes although a number of other
strains and species are also available. Yeast vectors typically consist
of a replication origin (unless of the integrating type), a selection
gene, a promoter, DNA encoding the receptor or its fragments, and

30   sequences for translation termination, polyadenylation, and
transcription termination.

Suitable expression vectors for yeast include such constitutive
promoters as 3-phosphoglycerate kinase and various other
glycolytic enzyme gene promoters or such inducible promoters as

35   the alcohol dehydrogenase 2 promoter or metallothionine pr moter.
Suitable vectors include derivatives of the following types: self-

replicating low copy number (such as the YRp-series), self-replicating high copy number (such as the YEp-series); integrating types (such as the YIp-series), or mini-chromosomes (such as the YCp-series).

5   Higher eukaryotic tissue culture cells are normally the preferred host cells for expression of the functionally active interleukin protein.   In principle, any higher eukaryotic tissue culture cell line is workable, e.g., insect baculovirus expression systems, whether from an invertebrate or vertebrate source.
10   However, mammalian cells are preferred. · Transformation or transfection and propagation of such cells has become a routine procedure.   Examples of useful cell lines include HeLa cells, Chinese hamster ovary (CHO) cell lines, baby rat kidney (BRK) cell lines, insect cell lines, bird cell lines, and monkey (COS) cell lines.
15   Expression vectors for such cell lines usually include an origin of replication, a promoter, a translation initiation site, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a transcription termination site.   These vectors also usually contain a selection gene or amplification gene.   Suitable expression vectors
20   may be plasmids, viruses, or retroviruses carrying promoters derived, e.g., from such sources as from adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus.   Representative examples of suitable expression vectors include pCDNA1; pCD, see Okayama *et al., Mol. Cell Biol. 5*:1136 (1985); pMC1neo PolyA, see
25   Thomas *et al., Cell 51*:503 (1987); and a baculovirus vector such as pAC 373 or pAC 610.

          For secreted proteins, an open reading frame usually encodes a polypeptide that consists of a mature or secreted product covalently linked at ist N-terminus to a signal peptide.   The signal peptide is
30   cleaved prior to secretion of the mature, or active, polypeptide.   The cleavage site can be predicted with a high degree of accuracy from empirical rules, e.g., von Heijne, *Nucleic Acids Research 14*:4683 (1986), and the precise amino acid composition of the signal peptide does not appear to be critical to its function, e.g., Randall *et al.,*
35   *Science 243*:1156 (1989); Kaiser *et al., Science 235*:312 (1987).

It will often be desired to express these polypeptides in a system which provides a specific or defined glycosylation pattern. In this case, the usual pattern will be that provided naturally by the expression system. However, the pattern will be modifiable by exposing the polypeptide, e.g., an unglycosylated form, to appropriate glycosylating proteins introduced into a heterologous expression system. For example, the interleukin gene may be co-transformed with one or more genes encoding mammalian or other glycosylating enzymes. Using this approach, certain mammalian glycosylation patterns will be achievable in prokaryote or other cells.

The source of human IL-13 can be a eukaryotic or prokaryotic host expressing recombinant huIL-13 DNA, such as is described above. The source can also be a cell line such as mouse Swiss 3T3 fibroblasts, but other mammalian cell lines are also contemplated by this invention, with the preferred cell line being from the human species.

Now that the entire sequence is known, human IL-13, fragments, or derivatives thereof can be prepared by conventional processes for synthesizing peptides. These include processes such as are described in Stewart et al., Solid Phase Peptide Synthesis, 1984, Pierce Chemical Co., Rockford, IL; Bodanszky et al., The Practice of Peptide Synthesis, 1984, Springer-Verlag, New York; and Bodanszky, The Principles of Peptide Synthesis, 1984, Springer-Verlag, New York. For example, an azide process, an acid chloride process, an acid anhydride process, a mixed anhydride process, an active ester process (for example, p-nitrophenyl ester, N-hydroxysuccinimide ester, or cyanomethyl ester), a carbodiimidazole process, an oxidative-reductive process, or a dicyclohexylcarbodiimide (DCCD)/additive process can be used. Solid phase and solution phase syntheses are both applicable to the foregoing processes.

The IL-13 protein, fragments, or derivatives are suitably prepared in accordance with the above processes as typically employed in peptide synthesis, generally either by a so-called stepwise process which comprises condensing an amino acid to the terminal amino acid, one by one in sequence, or by coupling peptide

fragments to the terminal amino acid. Amino groups that are not
being used in the coupling reaction typically must be protected to
prevent coupling at an incorrect location.

   If a solid phase synthesis is adopted, the C-terminal amino
5 acid is bound to an insoluble carrier or support through its carboxyl
group. The insoluble carrier is not particularly limited as long as it
has a binding capability to a reactive carboxyl group. Examples of
such insoluble carriers include halomethyl resins, such as
chloromethyl resin or bromomethyl resin, hydroxymethyl resins,
10 phenol resins, tert-alkyloxycarbonylhydrazidated resins, and the
like.

   An amino group-protected amino acid is bound in sequence
through condensation of its activated carboxyl group and the
reactive amino group of the previously formed peptide or chain, to
15 synthesize the peptide step by step. After synthesizing the complete
sequence, the peptide is split off from the insoluble carrier to
produce the peptide. This solid-phase approach is generally
described by Merrifield *et al.*, in *J. Am. Chem. Soc.* 85:2149 (1963).

   The prepared protein and fragments thereof can be isolated
20 and purified from the reaction mixture by means of peptide
separation, for example, by extraction, precipitation, electrophoresis,
various forms of chromatography, and the like. The interleukin of
this invention can be obtained in varying degrees of purity
depending upon its desired use. Purification can be accomplished by
25 use of the protein purification techniques disclosed herein or by the
use of the antibodies herein described in methods of
immunoabsorbant affinity chromatography. This immunoabsorbant
affinity chromatography is carried out by first linking the antibodies
to a solid support and then contacting the linked antibodies with
30 solubilized lysates of appropriate cells, lysates of other cells
expressing the interleukin, or lysates or supernatants of cells
producing the protein as a result of DNA techniques, see below.

   Generally, the purified protein will be at least about 40% pure,
ordinarily at least about 50% pure, usually at least about 60% pure,
35 typically at least about 70% pure, more typically at least about 80%
pure, preferable at least about 90% pure and more preferably at

least about 95% pure, and in particular embodiments, 97%-99% or more. Purity will usually be on a weight basis, but can also be on a molar basis. Different assays will be applied as appropriate.

VI. Antibodies

Antibodies can be raised to the various human IL-13 proteins and fragments thereof, both in naturally occurring native forms and in their recombinant forms, the difference being that antibodies to the active ligand are more likely to recognize epitopes which are only present in the native conformations. Anti-idiotypic antibodies are also contemplated, which would be useful as agonists or antagonists of a natural receptor or an antibody.

Antibodies, including binding fragments and single chain versions, against predetermined fragments of the protein can be raised by immunization of animals with conjugates of the fragments with immunogenic proteins. Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to normal or defective protein, or screened for agonistic or antagonistic activity. These monoclonal antibodies will usually bind with at least a K_D of about 1 mM, more usually at least about 300 μM, typically at least about 100 μM, more typically at least about 30 μM, preferably at least about 10 μM, and more preferably at least about 3 μM or better.

The antibodies, including antigen binding fragments, of this invention can have significant diagnostic or therapeutic value. They can be potent antagonists that bind to the interleukin and inhibit binding to the receptor or inhibit the ability of huamn IL-13 to elicit a biological response. They also can be useful as non-neutralizing antibodies and can be coupled to toxins or radionuclides to bind producing cells, or cells localized to the source of the interleukin. Further, these antibodies can be conjugated to drugs or other therapeutic agents, either directly or indirectly by means of a linker.

The antibodies of this invention can also be useful in diagnostic applications. As capture or non-neutralizing antibodies, they can bind to the interleukin without inhibiting receptor binding.

As neutralizing antibodies, they can be useful in competitive binding assays. They will also be useful in detecting or quantifying IL-13.

Protein fragments may be joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides
5   to be used as immunogens. The human IL-13 and its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See *Microbiology*, Hoeber Medical Division, Harper and Row, 1969; Landsteiner, *Specificity of Serological Reactions*, 1962,
10   Dover Publications, New York; and Williams *et al.*, *Methods in Immunology and Immunochemistry*, 1967, Vol. 1, Academic Press, New York, for descriptions of methods of preparing polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected
15   shortly after the repeated immunizations and the gamma globulin is isolated.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such
20   monoclonal antibodies may be found in, e.g., Stites *et al.* (eds), *Basic and Clinical Immunology* (4th ed.), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow *et al.*, *Antibodies: A Laboratory Manual*, 1988, CSH Press; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed), 1986, Academic Press, New York;
25   and particularly in Kohler and Milstein, *Nature 256*:495 (1975), which discusses one method of generating monoclonal antibodies.

Briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells. The result is a
30   hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells
35   from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Other suitable techniques involve *in vitro* exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See, Huse *et al., Science 246*:1275 (1989); and Ward *et al., Nature* 341:544 (1989). The polypeptides and antibodies of the present invention may be used with or without modification, including chimeric or humanized antibodies.

Frequently, the polypeptides and antibodies will be labelled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant or chimeric immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567.

The antibodies of this invention can also be used for affinity chromatography in isolating the IL-13. Columns can be prepared where the antibodies are linked to a solid support, e.g., particles, such as agarose, Sephadex, or the like, where a cell lysate may be passed through the column, the column washed, followed by increasing concentrations of a mild denaturant, whereby the purified protein will be released.

The antibodies may also be used to screen expression libraries for particular expression products. Usually the antibodies used in such a procedure will be labelled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against each human IL-13 will also be used to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various immunological conditions related to expression of the protein or cells which express receptors for the protein. They also will be useful as agonists or antagonists of the interleukin, which may be competitive inhibitors or substitutes for naturally occurring ligands.

VII.   Uses of IL-13 Compositions. Nucleic acids

Both naturally occurring and recombinant forms of the human
Interleukin-13 molecules of this invention are particularly useful in
kits and assay methods.   For example, these methods would also be
5   applied to screening for binding activity to these proteins.   Several
methods of automating assays have been developed in recent years
so as to permit screening of tens of thousands of compounds per
year.   See, e.g, a BIOMEK automated workstation, Beckman
Instruments, Palo Alto, California, and Fodor *et al., Science 251*:767
10   (1991).   The latter describes means for testing binding by a plurality
of defined polymers synthesized on a solid substrate.   The
development of suitable assays to screen for a receptor or
agonist/antagonist homologous proteins can be greatly facilitated by
the availability of large amounts of purified, soluble interleukin in
15   an active state such as is provided by this invention.

Rational drug design may also be based upon structural
studies of the molecular shapes of a receptor or antibody and other
effectors or ligands.   Effectors may be other proteins which mediate
other functions in response to ligand binding, or other proteins
20   which normally interact with the receptor.   One means for
determining which sites interact with specific other proteins is a
physical structure determination, e.g., x-ray crystallography or 2
dimensional NMR techniques.   These will provide guidance as to
which amino acid residues form the molecular contact regions.   For a
25   detailed description of protein structural determination, see, e.g.,
Blundell *et al., Protein Crystallography*, (1976) Academic Press, New
York.

Purified interleukin-13 can be coated directly onto plates for
use in the aforementioned receptor screening techniques.   However,
30   non-neutralizing antibodies to these proteins can be used as capture
antibodies to immobilize the respective interleukin on the solid
phase, useful, e.g., in diagnostic uses.

This invention also contemplates use of interleukin-13,
fragments thereof, peptides, and their fusion products in a variety of
35   diagnostic kits and methods for detecting the presence of the protein

or its receptor. Alternatively, or additionally, antibodies against the molecules may be incorporated into the kits and methods. Typically the kit will have a compartment containing either a defined IL-13 peptide or gene segment or a reagent which recognizes one or the

5   other. Typically, recognition reagents, in the case of peptide, would be a receptor or antibody, or in the case of a gene segment, would be a probe.

A preferred kit for determining the concentration of, for example, IL-13, a sample would typically comprise a labelled

10   compound, e.g., receptor or antibody, having known binding affinity for IL-13, a source of IL-13 (naturally occurring or recombinant) as a positive control, and a means for separating the bound from free labelled compound, for example a solid phase for immobilizing the IL-13 in the test sample. Compartments containing reagents, and

15   instructions, will normally be provided.

Antibodies, including antigen binding fragments, specific for IL-13 or a peptide fragment, or receptor fragments are useful in diagnostic applications to detect the presence of elevated levels of IL-13 and/or its fragments. Diagnostic assays may be homogeneous

20   (without a separation step between free reagent and antibody-antigen complex) or heterogeneous (with a separation step). Various commercial assays exist, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), enzyme-multiplied immunoassay technique (EMIT), substrate-

25   labelled fluorescent immunoassay (SLFIA) and the like.

For example, unlabelled antibodies can be employed by using a second antibody which is labelled and which recognizes the antibody to IL-13 or to a particular fragment thereof. These assays have also been extensively discussed in the literature. See, e.g.,

30   Harlow *et al., Antibodies: A Laboratory Manual*, 1988, CSH; and Coligan (Ed.), *Current Protocols In Immunology*, 1991 and periodic supplements, Greene/Wiley, New York.

Anti-idiotypic antibodies may have similar use to serve as agonists or antagonists of IL-13. These should be useful as

35   therapeutic reagents under appropriate circumstances.

Frequently, the reagents for diagnostic assays are supplied in kits, so as to optimize the sensitivity of the assay. For the subject invention, depending upon the nature of the assay, the protocol, and the label, either labelled or unlabelled antibody, or labelled receptor is provided. This is usually in conjunction with other additives, such as buffers, stabilizers, materials necessary for signal production such as substrates for enzymes, and the like. Preferably, the kit will also contain instructions for proper use and disposal of the contents after use. Typically the kit has compartments for each useful reagent. Desirably, the reagents are provided as a dry lyophilized powder, where the reagents may be reconstituted in an aqueous medium having appropriate concentrations for performing the assay.

Any of the constituents of the diagnostic assays may be used without modification or may be modified in a variety of ways. For example, labelling may be achieved by covalently or non-covalently joining a moiety which directly or indirectly provides a detectable signal. In any of these assays, a test compound, IL-13, or antibodies thereto can be labelled either directly or indirectly. Possibilities for direct labelling include label groups: radiolabels such as ^{125}I, enzymes (U.S. Pat. No. 3,645,090) such as peroxidase and alkaline phosphatase, and fluorescent labels (U.S. Pat. No. 3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization. Possibilities for indirect labelling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups.

There are also numerous methods of separating the bound from the free ligand, or alternatively the bound from the free test compound. The IL-13 can be immobilized on various matrixes followed by washing. Suitable matrices include plastic such as an ELISA plate, filters, and beads. Methods of immobilizing the receptor to a matrix include, without limitation, direct adhesion to plastic, use of a capture antibody, chemical coupling, and biotin-avidin.

The last step in this approach involves the precipitation of antibody/antigen complex by any of several methods including those utilizing, e.g., an organic solvent such as polyethylene glycol or

a salt such as ammonium sulfate. Other suitable separation techniques include, without limitation, the fluorescein antibody magnetizable particle method described in Rattle *et al.*, *Clin. Chem.* *30*:1457 (1984), and the double antibody magnetic particle separation as described in U.S. Pat. No. 4,659,678.

The methods for linking protein or fragments to various labels have been extensively reported in the literature and do not require detailed discussion here. Many of the techniques involve the use of activated carboxyl groups either through the use of carbodiimide or active esters to form peptide bonds, the formation of thioethers by reaction of a mercapto group with an activated halogen such as chloroacetyl, or an activated olefin such as maleimide, for linkage, or the like. Fusion proteins will also find use in these applications.

Another diagnostic aspect of this invention involves use of oligonucleotide or polynucleotide sequences taken from the sequence of an IL-13. These sequences can be used as probes for detecting levels of the IL-13 in patients suspected of having a proliferative cell conditions, e.g., cancer. The preparation of both RNA and DNA nucleotide sequences, the labelling of the sequences, and the preferred size of the sequences has received ample description and discussion in the literature.

Normally an oligonucleotide probe should have at least about 14 nucleotides, usually at least about 18 nucleotides, and the polynucleotide probes may be up to several kilobases. Various labels may be employed, most commonly radionuclides, particularly ^{32}P. However, other techniques may also be employed, such as using biotin modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labelled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like.

Alternatively, antibodies may be employed which can recognize specific duplexes, including DNA duplexes, RNA duplexes, DNA-RNA hybrid duplexes, or DNA-protein duplexes. The antibodies in turn may be labelled and the assay carried out where the duplex is bound t a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be

48

detected. The use of probes to the novel anti-sense RNA may be carried out in any conventional techniques such as nucleic acid hybridization, plus and minus screening, recombinational probing, hybrid released translation (HRT), and hybrid arrested translation

5   (HART). This also includes amplification techniques such as polymerase chain reaction (PCR).

Diagnostic kits which also test for the qualitative or quantitative presence of other markers are also contemplated. Diagnosis or prognosis may depend on the combination of multiple

10   indications used as markers. Thus, kits may test for combinations of markers. See, e.g., Viallet *et al., Progress in Growth Factor Res.* 1:89 (1989).

VIII. Therapeutic Utility

This invention provides reagents with significant therapeutic

15   value. The IL-13 (naturally occurring or recombinant), fragments thereof, and antibodies thereto, along with compounds identified as having binding affinity to the interleukin or its receptor or antibodies, should be useful in the treatment of conditions exhibiting abnormal expression of the interleukin. Such abnormality will

20   typically be manifested by immunological disorders. Additionally, this invention should provide therapeutic value in any disease or disorder associated with abnormal expression or abnormal triggering of response to the interleukin.

Recombinant IL-13 or IL-13 antibodies can be purified and

25   then administered to a patient. These reagents can be combined for therapeutic use with additional active ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, along with physiologically innocuous stabilizers and excipients. These combinations can be sterile filtered and placed into dosage forms as

30   by lyophilization in dosage vials or storage in stabilized aqueous preparations. This invention also contemplates use of antibodies or binding fragments thereof which are not complement binding.

Receptor screening using IL-13 or fragments thereof can be performed to identify molecules having binding affinity to the

interleukin. Subsequent biological assays can then be utilized to determine if a receptor can provide competitive binding, which can block intrinsic stimulating activity. Receptor fragments can be used as a blocker or antagonist in that it blocks the activity of IL-13.

5   Likewise, a compound having intrinsic stimulating activity can activate the receptor and is thus an agonist in that it simulates the activity of IL-13. This invention further contemplates the therapeutic use of antibodies to IL-13 as antagonists.

     The quantities of reagents necessary for effective therapy will

10   depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for in situ

15   administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman *et al.* (eds), *The Pharmacological Bases of Therapeutics*, 8th Ed., 1990, Pergamon Press; and *Remington's*

20   *Pharmaceutical Sciences*, 17th ed., 1990, Mack Publishing Co., Easton, Penn.

     Methods for administration are discussed therein and below, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically

25   acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the *Merck Index*, Merck & Co., Rahway, New Jersey.

     Because of the likely high affinity binding between an IL-13 and its receptors, low dosages of these reagents would be initially

30   expected to be effective. Thus, dosage ranges would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 μM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an

35   appr priate carrier. Slow release formulations, or slow release apparatus will often be utilized for continuous administration.

IL-13 or fragments thereof, antibodies or fragments thereof, antagonists, and agonists, may be administered directly to the host to be treated or, depending on the size of the compounds, it may be desirable to conjugate them to carrier proteins such as ovalbumin or

5   serum albumin prior to their administration. Therapeutic formulations may be administered in any conventional dosage formulation.

While it is possible for the active ingredient to be administered alone, it is preferable to present it as a pharmaceutical

10 formulation. Formulations comprise at least one active ingredient, as defined above, together with one or more acceptable carriers thereof. Each carrier must be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations

15 include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration.

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in

20 the art of pharmacy. The therapy of this invention may be combined with or used in association with other immunotherapeutic or immunopreventive agents.

EXAMPLES

The broad scope of this invention is best understood with

25 reference to the following examples, which are not intended to limit the inventions in any manner. Unless otherwise specified, percentages given below for solids in solid mixtures, liquids in liquids, and solids in liquids are on a wt/wt, vol/vol and wt/vol basis, respectively. Unless defined otherwise, all technical and

30 scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

Many techniques applicable to IL-4 and IL-10 may be applied to IL-13, as described, e.g., in U.S. Patent No. 5,017,691 (IL-4) and U.S.S.N. 07/453,951 (IL-10).

I. **Isolation of cDNA Clone Encoding Human IL-13**

5   An approximately 400 bp DNA fragment derived from a Pst/PvuII restriction digest of the mouse P600 cDNA clone was isolated by polyacrylamide gel electrophoresis and subsequent elution and ethanol precipitation. This fragment, which encompasses most of the coding region of the mouse P600 cDNA,

10   was radioactively labelled by random priming in the presence of [^{32}P]dCTP.

Filter lifts were prepared following standard procedures from ten agar plates each with approximately 5000 colonies of a B21 cDNA library. This library was made from cloned human T cells,

15   designated B21, which had been stimulated with anti-CD3 for 7 hours prior to the isolation of the RNA. The construction of this library is described in U.S. application Serial No. 07/453,951.

The filters were hybridized overnight at 42°C with the labelled mouse P600 fragment in 20% formamide, 6X SSPE, 0.1% SDS, 5X

20   Denhardt's solution, and 100 μg/ml tRNA. The filters were washed 3 times with 2X SSPE, 0.1% SDS at room temperature for 20 minutes each, twice with 1X SSPE, 0.1% SDS at 55°C for 1 hour, then exposed to film overnight. Eight positives were identified and picked for further purification. Seven of these were positive upon rescreening.

25   Six clones had *Bam*H1 inserts of 1.35 kb and one an insert of only 0.6 kb. The 1.35 kb insert of one clone, designated pB21.2Bf, was subcloned into M13 and sequenced by the dideoxy method. Sequence comparision demonstrated that this 1.16 kb cDNA encodes a human homolog of mouse P600.

30   The huIL-13 cDNA isolated from the B21 library was not full length as compared to the mouse P600 cDNA. Repeated attempts to isolate a full-length cDNA from the B21 library were unsuccessful. Thus, a different library was screened with the pB21.2Bf insert for a

full length clone. A PCR probe was derived from the human cDNA beginning 50 bp from the 5' end and ending at the stop codon.

A cDNA library was made from a clone of an A10 T cell line. The same hybridization conditions as described above were used. The filters were washed once in 1X SSPE, 0.05% SDS at room temperature for 15 minutes and then twice at 55°C for 30 min to one hour. They were exposed to film overnight. Several positives colonies were detected and rescreened. Double stranded sequence obtained from the 5' end of several of the cDNA inserts from these positives indicated that they were full-length. One of the 1.3 kb cDNA inserts, from a clone designated pA10.66, was subcloned into M13 and sequenced. Its sequence is shown in Table 1. The sequence of the full length clone differed from the sequence shorter clone by a single codon, which is present in the full length clone, see Table 3.

II. Expression and Purification of Mouse P600 and Human IL-13 Protein

The pB21.2Bf clone, containing a 1.16kb cDNA encoding human IL-13, lacked the first 23 N-terminal amino acids. The insert was prepared for ligation into an expression vector pGEX-2T by using PCR to provide unique restriction sites at the 5' *Bam*H1 and 3' *Eco*R 1 ends. The pGEX-2T vector is designed to produce a fusion protein, where the distinct protein segments are separated by a readily cleaveable protease site. The gel purified DNA was ligated into the expression vector pGEX-2T so that when the plasmid is expressed in *E. coli* the protein encoded by the DNA insert produces a fusion protein with glutathione-S-transferase with a thrombin cleavage site in between as described in detail by Smith *et al., Gene 67*:31 (1988). The resulting plasmid was transformed into *E. coli* and successful transformants were grown in the presence of IPTG. Expression products of this construct, when grown under inducing conditions, accumulated in inclusion bodies.

Mouse P600 Refolding and Purification

Transformed *E. coli* cells were grown in media at 37°C and
induced with IPTG at 0.5 OD. The induced cells were grown until
maximal OD was reached either by shake flask or fermentation.
5   Cells were harvested by centrifugation at 4000 x g at 4°C for 30 min
and were frozen at -10°C.

Cells were resuspended at room temperature in TE buffer (50
mM Tis-HCl, 10 mM EDTA pH 8 with 1 mM Peflobloc, a protease
inhibitor). Cells were passed through a microfluidizer at 18,000 psi,
10   collected, and centrifuged at 10,000 x g at 4°C for 30 min. Cell
pellets were repeatedly washed in TE buffer and centrifuged until
supernatant was clear. The pellet was solubilized with 6 M
guanidine-HCl, 10 mM DTT, 50 mM Tris-HCl pH 9, and 1 mM
Peflobloc and mixed at 4°C for 2 hours.

15   The protein concentration was measured by the Bradford
Protein method and typically was approximately 2.5 mg/ml total
protein concentration. The mixture was diluted over a period of
hours to 100 fold of its unfolding volume into 50 mM Tris-HCl, 150
mM NaCl, 2 mM reduced glutathione, 1 mM oxidized glutathione, 0.5
20   M guanidine-HCl, and 10 mM EDTA at pH 9.0. The solution was
mixed at 4°C for 24 hours, allowing refolding of disulfide linkages of
the molecule.

Precipitates were removed by centrifugation at 4000 x g for
30 min at 4°C or by filtration with a 0.45 μm filter. The supernatant
25   was concentrated using a Pellicon and diafilter against 50 mM TRIS-
HCL, 150 mM NaCl, 2.5 mM CaCl₂, pH 7.5 buffer at 4°C. The
glutathione-S-transferase fusion partner was cleaved by adding
human thrombin at 10 ng per 50 μg of fusion protein. The solution
was mixed at 4°C for 18 to 48 h to allow the fusion protein to be
30   cleaved by thrombin. SDS-PAGE gels and TF-1 bioassay was used to
characterize P600 conformation and activity.

Ammonium sulfate was aded to the refolded material at a 25%
saturation range after complete thrombin cleavage was observed.
The refolded material was adjusted to pH 8.5 with 6N NaOH and
35   loaded onto a butyl Toyo Pearl column, equilibrated at pH 8.5 with

25% ammonium sulfate, 50 mM TRIS-HCL, 0.05 M NaCl buffer. The
column was washed with equilibration buffer until the $\overset{\circ}{A}280$
approached baseline. The colmn was eluted with 50 mM TRIS pH
8.5 buffer for 5 bed volumes. An $\overset{\circ}{A}280$ pool was collected and
5   concentrated in a 5000 molecular weight cutoff Amicon stir cell to a
volume less than 3% of the bed volume of the S200 gel filtration
column.

The S-200 column was equilibrated with depyrogenated
buffer 50 mM NaPi, 150 mM NaCl, and 0.01% Tween-20 pH 6.0. The
10   concentrated S pool was loaded onto the gel filtration column and
fractions were collected and verified for P600 protein content by
SDS-PAGE gels. Fractions were pooled based on SDS-PAGE,
concentrated, filtered through 0.22 μm filters and tested by the TF-1
bioassay for biological activity. Protein concentration was
15   determined by silver staining and scan using the Molecular Dynamic
gel scanner.

Endotoxin was measured using the Whittaker colorimetric
Limulus assay and typically was < 10 eu/ml typical preparations
resulted in > 95% purity by staining, with a bioactivity of about 1 x
20   10^6 units/ml. Variations on the refolding procedure will be
effective, e.g., protein concentrations may vary over some range,
typically a 5-fold difference will also work. The glutathione
concentrations may be varied, and the periods of time for slow
dilution and overnight incubations may be titrated. Each of the
25   refolding parameters described should be titrated where
appropriate.

Similar procedures were used prepare and to purify human
IL-13.

III. Activities on B Cells

30   A. Cofactor/factor Proliferation: Cell Viability

Mouse P600 functions as a stimulator/costimulator of cell
viability, e.g., mouse P600 made from *E. coli* can stimulate or
costimulate proliferation of large *in vivo* activated mouse   B cells.

Decreasing amounts of P600 administered to the cells resulted in
lessened cell growth as determined by ^{3}H-thymidine incorporation.

To construct a cDNA encoding the extracellular domain of CD40
(designated "soluble CD40"), the following PCR primers containing an
5   *Xho*I site were synthesized on an Applied Biosystems 380A DNA
synthesizer:

sense: 5'-ACAGCTCGAGCCATG- GTGTCTTTGCCTCGGCTGTG-3' and
antisense: 5'-GTAGCTCGAGCTCACCGGGACTTTAAACCACAGATG-3'.

These primers were used to produce PCR fragments encoding
10   191 amino acids from the start codon of mouse CD40. PCR fragments
were digested with *Xho*I and then ligated into *Xho*I cleaved
mammalian/bacterial expression vector (pME18S).   The inserted
fragment was sequenced by the dideoxy sequenceing method to
confirm the sequence.
15   Plasmids carrying the soluble CD40 cDNA were transfected into
COS-7 cells by electroporation by standard procedures, See Ausubel
et al. (1987 and periodic supplements).   Briefly, 0.75 ml of COS-7 cell
suspension in serum free Dulbecco's Minimal Essential (DME)
medium at 10^7 cells per ml were incubated with 50 μl of 20 μg
20   plasmid at room temperature for 10 min, and subjected to
electroporation using a Bio-Rad gene pulser (960 F, 220 V).   Ten
minutes after electroporation, COS-7 cells were cultured in four 10
cm dishes for 3 days. For the purification of soluble CD40, medium
was changed to phenol red-free RPMI 1640 supplemented with
25   HB101 (HANA Biologics, Alameda, CA) one day after electroporation.
Soluble CD40 was purified by ion exchange chromatography on
anion exchange columns using standard procedures.   The protein
was eluted from the column using a linear NaCl gradients and
analyzed by Western Blotting using a rabbit antiserum against a
30   CD40 peptide made by standard methods.

Eight week old female Lewis rats were obtained from Harlan
Sprague-Dawley (Indianapolis, IN). These rats were immunized
intaperitoneally with 10 μg of soluble CD40 in complete Freund's

adjuvant followed by boosts of 10, 10, 10, and 50 μg of soluble CD40
in incomplete Freund's adjuvant at 3, 4.5, 6, and 8.5 weeks,
respectively. A final boost in saline was injected at 12 weeks. Test
bleeds were evaluated for anti-CD40 antibody content by ELISA.

5   Small dense B cells from unstimulated mouse spleens were
prepared as described in Hodgkin *et al., Cell. Immunol. 134*:14
(1991). Spleens were teased into complete RPMI (cRPMI) containing
5% fetal calf serum (FCS; J.R. Scientific, Woodland, CA), 5 x 10^{-5} M
2-mercaptoethanol (Polysciences, Inc., Warrington, PA), 2 mM
10   glutamine (J.R. Scientific), and 25 mM HEPES buffer (Irvine
Scientific, Santa Ana, CA), 100 U/ml penicillin, and 100 μg/ml
streptomycin (Irvine). Red blood cells were lysed using 0.83%
ammonium chloride, pH 7.4.

        T cells were removed using two successive treatments with
15   anti-mouse Thy 1.2 mAb (New England Nuclear, Boston, MA) and
anti-L3T4 antibody (RL172.4 hybridoma, a gift from Dr. H.R.
MacDonald, Ludwig Institute, Epalinges, Switzerland) for 20 min on
ice followed by complement (1:10 dilution of rabbit low-tox
complement, Cedarlane Laboratory, Ontario, Canada) for 30 min at
20   37°C. Small dense B cells were then isolated by density gradient
centrifugation using a discontinuous gradient composed of 75%, 65%,
and 50% percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) at
2500 x g for 25 min at 4°C.

        Cells collected from the interface between 65% and 75% percoll
25   were used in subsequent experiments. Large *in vivo* activated B
cells were collected from the 65% and 50% interface. B cells were
cultured in flat bottomed 96 well tissue culture plates (3072, Falcon
Labware) at various cell densities in cRPMI, plus additional
stimulants, as indicated. Proliferation was evaluated via a 4 hr
30   pulse of ^{3}H-thymidine (Amersham) added at 48 hr after culture
initiation.

35

B. Sustained Survival of B Cells: Selectivity Reagents

The anti-CD40 mAb89 and anti-CD23 mAb25 were produced
by standard procedures against the respective antigens, see Vallé *et
al., Eur. J. Immunol. 19*:1463 (1989); Bonnefoy *et al., J. Immunol.*
5 *138*:2970 (1987). The CDw32/Fcγ RII transfected Ltk-cell line
(CDw32 L cells) was described by Peltz *et al., J. Immunol. 141*:1891
(1988). Anti-IgM antibodies coupled to beads (anti-M) were
purchased from Biorad (Richmond, CA).

Cell phenotype was determined using FITC conjugated mAb
10 originating from Becton Dickinson (Mountain View, CA). The
neutralizing anti-IL-4 monoclonal antibody was kindly provided by
Dr. Grassi. The extracellular domain of the 130 kDa IL-4 receptor
was derived from COS-7 cells transfected with a plasmid containing
a truncated IL-4 cDNA described by Garrone *et al., Eur. J. Immunol.*
15 *21*:1365 (1991). The recombinant protein was purified from
transfected cell culture supernatant by purification on an IL-4-Affi-
gel 10 column. The anti-130 kDa IL-4 receptor antibody was
generated after immunization of mice with the extracellular domain
of the IL-4 receptor. Cultures were carried out in modified
20 Iscove's medium.

B Cell Preparations and Cell Cultures

B cells were isolated from tonsils as described by Defrance *et
al., J. Immunol. 139*:1135 (1987). Briefly, after a rosetting step with
sheep red blood cells, non-rosetting cells were further incubated
25 with anti-CD2, anti-CD3, and anti-CD14 mAbs prior to negative
selection performed with magnetic beads coated with anti-mouse
IgG (Dynabeads, Dynal, Oslo, Norway). The isolated population
expressed > 98% CD19 or CD20 (B cells) and < 1% CD2 (T cells) or
CD14 (monocytes).

Assays With Antigen Receptor Activated B Cells

30

B lymphocytes, adjusted at 5 x 10^5 cells/ml, were stimulated
for 72 hours with insolubilized anti-IgM (5 μg/ml). A 16 hour pulse

with 1 μCi (^{3}H)TdR was usually performed at day 3 and 6. [^{3}H]-TdR
uptake was measured by standard liquid scintillation counting
techniques.

CD40 system

5   For proliferation assays, 2.5 x 10^4 purified B cells were
cultured in the presence of 2.5 x 10^3 irradiated (7000 rad) CDw32 L
cells and 0.5 μg/ml of anti-CD40 mAb89 in a final volume of 200 μl.
For Ig production, B cells were tested at 2.5 x 10^5 cells/ml.
Supernatants were harvested after 10 days and Ig levels were
10   determined by ELISA.

Isolation of sIgD$^\pm$ and sIgD$^-$ B Cell Populations

           Purified B lymphocytes were separated using a preparative
magnetic cell separation system (MACS, Becton-Dickinson), according
to the experimental procedure described in detail by Miltenyi *et al.*,
15   *Cytometry* *11*:231 (1990).   The separation based on sIgD expression
has been described earlier by Defrance *et al.*, *J. Exptl Med.* *175*:671
(1992). Purity of the sorted cell populations were > 99% for the
sIgD$^+$ B cell subpopulation, while < 1% of sIgD$^-$ B cell subpopulation
expressed sIgD, as assessed by fluorescence analysis using a
20   FACScan.

Cytokines

           Purified recombinant hIL-2 (Amgen, Thousand Oaks, CA, 3 x
10^6 U/ml), recombinant hIL-4 (Schering-Plough Research Institute,
Bloomfield, NJ, 1 x 10^7 U/mg), recombinant hIL-10 (Schering-Plough
25   Research Institute, Bloomfield, NJ, 1 x 10^7 U/ml) were respectively
used at 10 U/ml, 50 U/ml, and 100 ng/ml. IL-13 was expressed as
a fusion protein with glutathione-S-transferase using the pGEX-2T
vector (Pharmacia, Uppsala, Swede).
           A DNA fragment encoding hIL-13 residues 24-109 was
30   prepared by polymerase chain reaction (PCR) and cloned into the
*Bam*HI/*Eco*RI site of the vector. A DNA fragment encoding mIL-13
residues 19-109 was also prepared and cloned. The human and

mouse IL-13 fusion proteins were expressed as insoluble aggregates in *Escherichia coli*, extracted by centrifugation, solubilized, and subjected to a renaturation step, see van Kimmenade *et al., Eur. J. Biochem. 173*:109 (1988).

5   The refolded IL-13 was cleaved from the fusion partner by thrombin, purified by cation exchange (S-Sepharose FPLC, Pharmacia) and gel filtration (Sephacryl s-200 FPLC, Pharmacia) chromatography. The gel filtration column was calibrated with protein standards (Bio-Rad). Proteins were quantitated by SDS-
10   PAGE, silver staining (ISS), and scanning densitometry (Molecular Dynamics) with normalization to chicken egg lysozyme (Sigma, St. Louis, MO). Endotoxin (determined by the Limulus ameobocyte lysate assay (Whittaker Bioproducts, Inc.) was typically < 1 eu/ml.

15   **IL-13 Enhances the DNA Synthesis of B Cells Activated Through Their Antigen Receptor**

The DNA synthesis of highly purified human B lymphocytes activated through their antigen receptor with anti-IgM antibody is enhanced by recombinant cytokines, such as IL-2, IL-4, and IL-10. Recombinant murine IL-13 also enhanced, in a dose dependent
20   fashion, the day 3 DNA synthesis of human tonsillar B lymphocytes cultured in the presence of insolubilized anti-IgM antibody. The maximum stimulation was obtained for a concentration of 10-25 ng/ml of murine (or human) IL-13.

The stimulatory effect was lower than that of either IL-2 or
25   IL-4 but comparable to that of IL-10. As IL-4, but unlike IL-2, the co-stimulatory effect of IL-13 on anti-IgM activated B cells could be observed after 3 days of culture, and decreased to be virtually undetectable after 6 days.

IL-13 Acts As Growth Factor for B Cells Stimulated Through Their
30   **CD40 Antigen**

IL-13 was also assayed for its ability to enhance the proliferation of anti-CD40 activated B cells in comparison with IL-4 and IL-10. Thus, 2.5 x 10^4 purified tonsillar B lymphocytes were

cultured over CDw32 expressing L cells together with 0.5 μg/ml of an anti-CD40 antibody Mab 89 with or without increasing concentrations of IL-13. [^{3}H]-TdR incorporation was measured at day 6. Both murine and human IL-13 strongly enhanced anti-CD40

5   induced DNA synthesis. Maximum stimulation was reached between 3 and 30 ng/ml IL-13 and plateaued thereafter without demonstrating any inhibitory effect (even at 1000 ng/ml). Under these culture conditions, the half maximal stimulation was observed between 0.03 and 0.3 ng/ml in three independent experiments.

10   The growth stimulatory effects of IL-13 were then compared to those of IL-4 and IL-10. IL-13 activity was comparable to that of IL-4 and IL-10 when assayed early in the culture at day 6. The IL-13 stimulatory activity was particularly striking at day 9, where it surpassed that of IL-10 and more notably that of IL-4. IL-13 also

15   showed stimulatory effects at day 12 and again was more efficient than either IL-4 or IL-10. Cultures grown in the presence of IL-13 formed extremely tight clumps. Clumps were very difficult to dissociate and thus rendered extremely inaccurate the enumeration of viable lymphocytes during cell cultures. Nevertheless, cell

20   cultures were split every fifth day for up to 25 days, at which time the number of viable B lymphocytes had increased about 12 fold (estimated conservatively).

        Whether IL-13 could act in concert with IL-4 or IL-10 for maximal B cell proliferation was studied. Combination of an optimal

25   concentration of IL-4 and with increasing concentration of IL-13 resulted in a DNA synthesis which was comparable to that obtained with IL-4, thus indicating the lack of synergy and even additivity between these two cytokines. In contrast, combination of IL-13 and IL-10 resulted in an additivity of their stimulatory effects. The

30   additive effects of IL-13 and IL-10 on B cell proliferation were observed at all times tested. Taken together, these results indicated that IL-13 is a growth factor for human B lymphocytes.

35

<u>IL-13 Induces Anti-CD40 Activated B Cells to Secrete IgE</u>

In view of the powerful effects of IL-13 on the proliferation of
anti-CD40 activated B cells, culture supernatants were analysed for
their immunoglobulin content. Like IL-4 but unlike IL-10, IL-13
5 did not stimulate the production of IgM, IgG, and IgA in day 8
cultures of anti-CD40 activated B cells. However, IL-13 was able to
induce B cells to secrete IgE in a dose dependent fashion (Table 4).
IL-13 was comparable to IL-4 in its capacity to induce IgE. The
combination of IL-4 and IL-13 resulted in a production of IgE which
10 was either comparable or slightly enhanced when compared to that
obtained with IL-4 alone. IL-13 unable to induce either resting B
cells or anti-μ activated B cells to secrete IgE.

Table 4: IL-13 Induces Anti-CD40 Activated B Cells to Secrete IgE.
15

cytokine	IgG (μg/ml)	IgA (μg/ml)	IgM (μg/ml)	IgE (ng/ml)
none	1.2 ± 0.09	0.4 ± 0.04	0.08 ± 0.005	< bg
IL-4	1.4 ± 0.01	0.4 ± 0.005	0.2 ± 0.005	37 ± 1.1
IL-10	15.0 ± 1.9	12.0 ± 2	18.0 ± 0.6	< bg
IL-13	1.6 ± 0.4	0.4 ± 0.001	0.2 ± 0.04	25.2 ± 3.3

5×10^4 purified B cells were cultured for 10 days with 2.5×10^3
irradiated CDw32 L cells with the anti-CD40 mAb89 without or with
50 U/ml IL-4, 100 ng/ml IL-10, 30 ng/ml hIL-13. Ig levels
20 represent the mean ± SD values of quadruplicate determinations.
Representative of three experiments. (< bg = lower than 150 pg/ml
of IgE.)

<u>The IL-13 Target B Cell Subpopulation is More Restricted Than the IL-4 One</u>

25   To further compare the effects of IL-4 and IL-13, B cells
cultured in the CD40 system with either cytokine were phenotyped
after six days of culture. IL-13 is able to induce CD23 expression on
cultured B cells with an intensity comparable to that obtained with
IL-4. However, whereas IL-4 induced > 90% of the cultured B cells

to express CD23, IL-13 induced CD23 only on 40% of the B cell
population. In addition, whereas IL-4 was able to readily induce
CD23 on both resting and anti-M activated B cells, IL-13 was much
less efficient under these conditions.

5   The transferrin receptor expression was also analyzed on B
cells cultured for six days in the CD40 system with or without IL-4
or IL-13. All B cells expressed transferrin receptors (TfR), but two
populations could be clearly distinguished according to levels of
expression which were designated TfR low and TfR high. In the

10   CD40 system alone, 80% of the B cells were TfR low, and 5% TfR high.
In cultures grown with IL-13, 55% of the cells were TfR low and 40%
were TfR high. In cultures performed with IL-4, 10% of the cells
were TfR low and 85% were TfR high.

As sIgD$^+$ B cells consist of naive B cells, whereas sIgD$^-$ B cells

15   consist of a mixture of germinal center B cells and memory B cells,
the reactivity to IL-4, IL-10, and IL-13 of sIgD$^+$ and sIgD$^-$ B cells
was tested in the CD40 system. Both sIgD$^+$ and sIgD$^-$ B cells
proliferated strongly in response to   IL-4 and IL-40, as measured by
(^{3}H)TdR incorporation after six days of culture. In contrast, IL-13

20   preferentially enhanced the anti-CD40 induced DNA synthesis of
sIgD$^+$ cells. Furthermore, IL-13 and IL-4 were able to induce both
sIgD$^+$ and sIgD$^-$ B cells to secrete IgE.

Taken together, these results indicate that IL-13 acts
preferentially on sIgD$^+$ cells and thus acts on a B cell subpopulation

25   more restricted in size than that stimulated by IL-4.

The IL-13 Biological Effects Are Independent of IL-4

As IL-13 displays many of the biological effects of IL-4, it was
suspected that it might act either through an induction of IL-4
secretion or through binding to the IL-4 receptor. To address this

30   question, IL-13-induced B cell proliferation was tested in the
presence of three different IL-4 antagonists: (1) a neutralizing anti-
IL-4 monoclonal antibody; (2) a soluble extracellular domain of the
130 kDa IL-4R [see Garrone et al., Eur. J. Immunol. 21:1365 (1991)];
and (3) a blocking anti-130 kDa IL-4R monoclonal antibody. These

three antagonists blocked by 80-90% the effects of IL-4 on the proliferation of anti-CD40 activated B lymphocytes without affecting the proliferation induced by IL-13. These IL-4 antagonists also failed to block IL-13 induced CD23 expression and IgE production,
5 while they did totally block that induced by IL-4.

Induction of B Cell Proliferation By IL-13 or IL-4 and Cos Cells Expressing the Human or Mouse CD40-L

Five x 10^4 highly purified (> 98% CD20+) negatively sorted splenic B cells were co-cultured with 1.6 x 10^4 irradiated (7,000
10 rad) Cos cells transfected with human or mouse CD40-L or the empty pJFE-14 vector as control. IL-13 or IL-4 were added at 400 U/ml. Soluble anti-CD40 mAB89 and the control mAbA4 were used at 50 μg/ml. The cultures were harvested 3 days later, after addition of [^{3}H]-Thymidine in the last 16 hours of culture. B cells
15 were stimulated by any of IL-4, COS supernatants containing either mouse or human IL-13, or combinations of supernatants and IL-4 or IL-13.

Biological effects of IL-13 on human B cell growth and differentiation have been described. IL-13 costimulated with anti-
20 IgM antibody to induce DNA synthesis but its effects were less conspicuous than those of IL-2 or IL-4. IL-13, as other cytokines, failed to greatly induce the multiplication of B cells activated through their antigen receptor. However, IL-13 displayed striking growth promoting effects on B cells which were cultured in the CD40
25 system, which is composed of a fibroblastic cell line expressing the human Fc receptor CDw32 and monoclonal antibody to CD40. Under these conditions, IL-13 was at least as active as IL-4 and its effects on B cells were long lasting, thus allowing the multiplication of viable B cells. IL-13 altered the phenotype of activated B cells, as it
30 induced B cells to express CD23.

The IL-13 dependent induction of CD23 on CD40 activated B cells is not mediated by an anti-CD40 activation, since resting and anti-IgM activated B cells can also be induced to express CD23 in response to IL-13. However, the proportion of cells expressing CD23
35 was lower with IL-13 than with IL-4. Likewise, IL-4 induced

virtually all CD40 activated B cells to express high levels of
transferrin receptors. IL-13 induced only half the cells to express
transferrin receptors at high density. This indicated that IL-13 was
acting on a subpopulation of B cells which was more restricted than
5   that affected by IL-4.

Accordingly, when cells were separated according to surface
IgD (sIgD), which distinguishes naive B cells from germinal center
and memory B cells, IL-13 was found to be more effective than IL-4
on sIgD+ B cells. IL-4 was able to enhance better than IL-13 the
10   proliferation of sIgD- B cells. The different population target for
IL-13 and IL-4 could be explained by differential IL-13 and IL-4
receptors expression, the demonstration of which will await the
production of labelled IL-13 or of IL-13 receptor specific antibodies.
The lower response of sIgD- B cells is particularly interesting and
15   warrants further analysis. IL-13, as IL-4, poorly enhanced the
synthesis of IgG and IgM by B cells cultured in the CD40 system.
Surprisingly, however, IL-13 induced anti-CD40 activated B cells to
produce IgE.

The levels of IgE produced in response to IL-13 were
20   comparable to those produced in response to IL-4. IL-4, as well as
IL-13, was able to induce IgE synthesis by anti-CD40 activated B
cells. As a consequence of isotype switching, IL-13, like IL-4, was
able to induce sIgD+ to secrete IgE. As IL-4, IL-13 could not induce
resting B cells to secrete IgE. At first, this IL-13 induced IgE
25   production contrasts with other studies indicating IL-4 as being the
sole inducer of IgE synthesis. However, recent studies have
described assay systems resulting in the secretion of IgE, while IL-4
was totally blocked by neutralizing antibody. IL-2 has been
reported to induce secretion by *Staphylococcus aureus* activated B
30   cells, whereas IL-4 was ineffective. It may be possible that in these
studies, IL-13 was responsible for these effects.

While IL-13 causes many functions similar to IL-4 on B cells,
the present study demonstrates that these effects are independent
of possible induction of IL-4 secretion or the use of the 130 kDa
35   IL-4 receptor, e.g., neutralizing anti-IL-4 antibody, blocking soluble
IL-4 receptor, and blocking anti-IL-4 receptor antibody were unable

to affect the action of IL-13 on B cell proliferation and IgE secretion. However, it cannot be excluded that IL-13 may share with IL-4 some common transducer.

Crosslinking studies have shown the binding of IL-4 to 60-70 and 70-80 kDa components unrelated to the 130 kDa molecule. In this context, it is worth noting that both human and murine IL-13 act on human B cells with similar efficiency, while murine IL-4 is species specific. The respective roles of IL-13 and IL-4 in IgE production can be determined more particularly when mice whose IL-4 genes have been knocked out (e.g., IL-4 knock-out mice) are studied to determine circulating IgE levels.

Finally, it will be important to establish whether human IL-13 is produced only by T cells or if other cell types also produce it. Furthermore, IL-13 may be involved in abnormal B cell proliferation, as occurs in leukemic and autoimmune diseases. Thus, agonists or antagonists of IL-13 may be useful in therapeutic treatment of such conditions.

C Modification of Ig Surface Markers on Activated Human B Cells

Highly purified B cells were isolated from normal human spleens obtained from cadaver transplant donors. Splenocytes were obtained by aseptically squashing spleens though a sterile metal mesh and frozen in aliquots for subsequent use. Highly purified B cells (> 98% CD20+) were obtained by negative FACStar Plus Becton Dickinson sorting after staining the splenocytes with the following PE-conjugated mAb: anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD16, and anti-CD56. (Becton Dickinson).

Human IL-13 was used at 30 ng/ml final concentration. Recombinant IL-4 (used at 400 U/ml) was provided by Schering Research (Bloomfield, NJ).

Five thousand highly purified B cells were co-cultured with an equal number of T cells from clones B21 or spA3, harvested five days after stimulation with feeder cells and PHA, in a final volume of 0.2 ml of Yssel's medium supplemented with 10% FCS, 10 μg/ml ultra pure transferrin (Pierce), and 400 U/ml recombinant IL-4. Cultures were set up in eight replicates in U-bottom 96 well plates

(Linbro) and incubated 14 days at 37°C in 5% CO_2. At the end of the
incubation period, the supernatants from each of the eight wells
were harvested and pooled for isotype determination. In some
cultures, the T cell clones were replaced by 5 µg of plasma
5   membranes derived from the T cell clones, or by 50 µg/ml anti-CD40
mAb 89.

Ig content of the supernatants was determined by ELISA as
described by Gascan *et al., Eur. J. Immunol.* 22:1133 (1992). Plasma
membranes were prepared from the CD4+ T cell clone B21, also as
10 described therein. The amounts of production of the respective Ig
isotypes were typically highest when B cells were stimulated with
membranes in the presence of IL-4, but IL-13 typically had similar
effects. The amount of IgG4 production was particularly high,
though IL-13 seemed to enhance the IL-4 effect on IgE production
15 after stimulation with T cell clones.

Highly purified sIgD+ splenic B cells (5000 cells/well) were co-
cultured with pJFE14 vector transfected or sorted COS-7 cells (250
cells/well) transfected with and expressing the human (h) or mouse
(m) CD40-L. IL-13 (30 ng/ml) and IL-4 (400 U/ml) were added.
20 The sensitivities of the ELISAs (0.2 ng/ml for IgE and IgM, 0.4
ng/ml for IgG4 and total IgG) were determined with calibrated Ig
standards (Behring, Marburg, Germany). Both IgG and IgE levels
were increased by IL-13.

D. Effects of CD40 Ligand

25 The human CD40 ligand (hCD40-L) was cloned from a cDNA
library constructed from an activated CD8+ T-cell clone and two
cDNA's were detected representing a 2.1 kb and a   1.2 kb clone.
Both cDNA clones had identical open reading frames of 261 amino
acids and differed only in the length of their 3' untranslated ends,
30 and probably represent the 2.1 kb and 1.2 kb transiently expressed
mRNA species detected by Northern analysis in an activated CD4+ T-
cell clone. hCD40-L transcripts could also be detected in CD4+ and
CD8+ T cell receptor (TCR) αβ T cells, TCR γδ T cells, natural killer
cells, monocytes, small intestine, and fetal thymocytes, but not in

purified B cells, fetal liver, fetal bone marrow, brain, kidney, or
heart.

COS-7 cells transfected with hCD40-L (COS-7/hCD40-L)
induced human B cell activation as judged by the induction of
5   homotypic aggregates of Epstein-Barr Virus (EBV) transformed, and
normal B cells. In addition, COS-7/hCD40-L induced B cell
proliferation, which was further enhanced by IL-4, or IL-13. IL-13,
like IL-4, synergized with the mouse and hCD40-L to induce IgM,
total IgG, IgG4, and IgE, but not IgA production by highly purified B
10   cells.

Anti-IL-4 antibodies inhibited IL-4 and COS-7/hCD40-L
induced Ig production by B cells, but had no effect on IL-13 and
COS-7/hCD40-L induced B cell differentiation, indicating that IL-13
and hCD40-L induced Ig production, including isotype switching to
15   IgE, independently of IL-4. hCD40-L induced B cell differentiation
was blocked by soluble CD40, confirming the requirement for
specific engagement of CD40-L. Collectively, these data indicate that
CD40-L and IL-13 expressed by human CD4$^+$ T helper cells are
important components of T and B cell interactions resulting in B cell
20   proliferation, differentiation, and IgE switching. However, the
distribution of the hCD40-L suggests a broader function of this
molecule.

Induction of B cell proliferation and differentiation into Ig
producing cells requires T cell help. Antigen-specific T cell and B
25   cell interactions involving binding of the TCR to peptide class II MHC
complexes in the B cells result in T cell activation. Activated T cells
deliver both contact and cytokine mediated signals, inducing B cell
proliferation and differentiation. Once T cells are activated, they can
interact with any B cell in an antigen independent class II MHC non-
30   restricted fashion. Lymphokines produced by the activated T helper
cells do not only determine the amounts of Ig produced, but they
also direct isotype switching.

IL-4 is a B cell growth factor which induces human B cells to
switch to IgG4 and IgE production, whereas TGF-β directs IgA
35   switching. The human cDNA homologue of P600, a protein produced
by mouse Th2 clones following activation, has been recently cloned

and expressed, as described herein. The human IL-13 protein
induced human monocyte and B cell growth and differentiation and
was designated IL-13. Human IL-13 is a non-glycosylated protein
of 132 amino acids with a molecular mass (M_r) of 10,000 and is
5   produced by T cells. IL-13 has no significant homology with other
cytokines except IL-4, which is ~30% homologous. IL-13, like IL-4,
can specifically induce IgG4 and IgE switching in human B cells,
independently of IL-4.

The contact mediated signals delivered by activate T helper
10   cells can be replaced by anti-CD40 mAbs. One of the contact T
helper signals is delivered by the CD40 ligand (CD40-L), a 33 kDa
molecule expressed on activated CD4$^+$ T cells. CD40-L transfectants
induced proliferation of B cells and induced IgE production in the
presence of IL-4. Here is described the isolation of human CD40-L
15   clones from a cDNA library constructed from an activated CD8$^+$ T-
cell clone. The distribution of the human CD40-L, its ability to
activate B cells, and its role as a co-activation molecule with IL-13
compared with IL-4 to differentiate B cells were assessed. Cells
transfected with the human CD40-L exhibited induced B cell
20   aggregation, proliferation, and considerable Ig production, including
IgE synthesis, in the presence of IL-13.

Reagents

Human rIL-4 was provided by Schering-Plough Research
(Bloomfield, NJ) and human rIL-13 was provided by W. Dang (DNAX
25   Research Institute, Palo Alto, CA). The CD40-Ig fusion protein was
obtained by fusion of the cDNA segments encoding the extracellular
domain of CD40 to cDNA fragments encoding the human IgG1. The
mAb89 was kindly provided by Dr. J. Banchereau (Schering-Plough,
Dardilly, France). Streptavidin-PE and all antibodies, unless stated
30   otherwise, were from Becton-Dickinson (Mountain View, CA).

Cell Purification and Culture

     B lymphocytes (>98% CD20+) were purified from spleen using density gradient centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ), followed by negative cell sorting using a

5 FACStar Plus (Becton Dickinson). Surface IgD+ positive cells were sorted directly from the negatively sorted B cell population. The CD4+ T-cell clone B21 and the CD8+ T-cell clone A10 have been described by Roncarolo *et al.*, *J. Exp. Med. 167*:1523 (1988). In co-culture experiments, various numbers of purified B cells were

10 cultured with different concentrations of COS-7 cells in U-bottom 96-well trays in 0.2 ml.

     After 10 days, 50% of the medium was replenished, and after 14 days the supernatants harvested and assayed for Ig's by ELISA. COS-7 cells were transiently transfected. For CD40-L staining, COS-7

15 or B21 cells were incubated on ice with 1.4 µg/ml biotinylated CD40-Ig in PBS, 1% FCS for 20 min, washed twice in PBS with 1% FCS, and stained with 1/5 dilution of streptavidin-PE, and washed twice again. Cells specifically expressing CD40-L were sorted using a FACStar Plus (Becton Dickinson) before use.

20 ## Human and Mouse CD40-L cDNA's

     Mouse CD40-L DNA provided as a PCR product by Dr. N. Harada and Dr. R. Chang (DNAX Research Institute) was subcloned into the mammalian expression vector pJFE14. The human CD40-L was cloned by using the mouse CD40-L cDNA as a probe to screen colony

25 blots of a cDNA library derived from the CD8+ T-cell clone A10. To make the library, 10^8 A10 cells were activated for 8 hours with 10 µg/ml con A, harvested, and extracted for RNA. mRNA was purified using a Pharmacia (Uppsala, Sweden) mRNA Purification Kit. cDNA was synthesized and cloned essentially according to the

30 manufacturers instructions using the SuperScript Plasmid System (BRL, Grand Island, NY) the only modification being the use of pJFE14 as the vector for cloning. The library contained 10^6 independent cl nes with an average insert size of 1.4 kb.

Northern and PCR Analysis

RNA was isolated using RNAzol B (CNNA: Biotech, Friendswood, TX) according to manufacturer's instructions. RNA from brain, heart, kidney, and small intestine were from Clontech (Palo Alto, CA).

5 cDNA was synthesized using SuperScript (BRL) and PCR reactions performed in a GeneAmp PCR System (Perkin-Elmer Cetus, Emeryville, CA) with 30 cycles of 94°C, 55°C, and 72°C for 0.5 min, 0.5 min, and 1 min respectively. Primers for detection of CD40-L transcripts were 5'-ACA GCA TGA TCG AAA CAT ACA-3', 5'- TGG CTC

10 ACT TGG CTT GGA TCA GTC-3' and for hypoxanthine phosphoribosyltransferase (HPRT) transcripts 5'-TAT GGA CAGGAC TGA ACG TCT TGC 3', 5' - GAGACA AACATGATT CAA ATCCCT GA-3'.

Products of PCR reactions were electrophoresed through 1.2% agarose and transferred by capillary blotting to GeneScreen nylon

15 membranes (NEN Research Products, Boston, MA) according to manufacturer's instructions. For Northern analysis RNA was electrophoresed through 0.85% agarose and transferred to BA-S nitrocellulose (Schleicher and Schuell, Keone, NH). CD40-L ^{32}P cDNA probes for Northern and Southern blots were made using as a

20 template a 1.3 kb EcoRI-XhoI fragment of pJFE14-CD40-L containing the CD40-L coding region.

Cloning and Characterization of the Human CD40-L

To obtain clones of the human CD40-L, a cDNA library derived from the CD8^{+} T-cell clone A10 was screened using the mouse CD40-L

25 cDNA as a probe, see Armitage et al., Nature 57:80 (1992).

Positive clones were present in the library at 0.005% and were represented predominantly by a 2.1 kb length clone, but an additional clone of 1.2 kb was detected. Both cDNA's contained an identical open reading frame of 261 amino acids, which would give

30 rise to a protein with an unmodified molecular mass of 29254. The 2.1 kb and 1.2 kb clones differed only in the length of their 3' untranslated ends, and presumably represent the two mRNA species of that size detected by Northern analysis. The nucleotide and predicted amino acid sequences of the human CD40-L cloned here

were identical to those reported by Hollenbaugh *et al., EMBO J.*
11:4313 (1992); and Spriggs *et al., J. Exp. Med. 176*:1543 (1992).

Using a biotinylated human CD40-Fc fusion protein in
combination with streptavidin-PE, specific expression could be easily

5   detected of human CD40-L on COS-7 cells transiently transfected
with an expression plasmid pJFE14 containing the 2.1 kb human
CD40-L cDNA, but not on control cells transfected with empty pJFE14
vector DNA.   The human CD40-Fc reagent reacted also with COS-7
cells transfected with the same expression vector containing the

10   mouse CD40-L cDNA, which is consistent with previous studies,
indicating cross-species binding of human CD40 to mouse CD40-L.

The CD40-L Induces Homotypic Aggregation of B Cells and B Cell Proliferation

B cell activation with antibodies to CD40 results in homotypic

15   aggregation.   To determine whether the CD40-L had similar effects,
COS-7 cells expressing the CD40-L were purified by FACS and co-
cultured with purified B cells or JY cells, an EBV transformed B cell
line.   Indeed, aggregation of JY cells following incubation with the
COS-7 cells expressing human or mouse CD40-L was observed,

20   whereas mock-transfected COS-7 cells were ineffective.   Similarly,
purified B cells co-cultured with cells expressing human or mouse
CD40-L displayed marked homotypic aggregations, whereas B cells
cultured with untransfected COS-7 cells remained disperse.

Consistent with the B cell activation observed microscopically,

25   significant proliferation was obtained when purified B cells were co-
cultured with COS-7/human CD40-L or COS-7/mouse CD40-L (see
Table 5).   This proliferation was further enhanced in the presence of
IL-4 or IL-13 .   The growth promoting effects of IL-4 and IL-13
seem to be comparable under these culture conditions.

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Table 5: Induction of B Cell Proliferation by IL-13 or IL-4 and COS
Cells Expressing the Human or Mouse CD40-L

	^{3}H TdR Incorporation (c.p.m. x 10^{-3})
B	0.1 ± 0
B + IL-13	0.1 ± 0
B + IL-4	0.2 ± 0
B + IL-4 + control mAb	0.2 ± 0
B + IL-4 + anti-CD40	21.2 ± 4.1
COS hCD40-L	1.1 ± 0.2
COS mCD40-L	1.0 ± 0.1
COS	1.4 ± 0.2
B + COS hCD40-L	16.9 ± 2.4
B + COS mCD40-L	17.5 ± 2.1
B + COS	1.2 ± 0.2
B + IL-4 COS hCD40-L	30.7 ± 3.8
B + IL-4 COS mCD40-L	35.4 ± 4.5
B + IL-4 COS	1.3 ± 0.2
B + IL-13 COS hCD40-L	22.5 ± 3.0
B + IL-13 COS mCD40-L	33.8 ± 2.8
B + IL-13 COS	1.2 ± 0.4

Five x 10^4 highly purified (> 98% CD20$^+$) negatively sorted splenic B
cells were co-cultured with 1.6 x 10^4 irradiated (7,000 rads) COS
cells transfected with human or mouse CD40-L or the empty pJFE-14
vector as control. IL-13 or IL-4 were added at 400 U/ml. Soluble
anti-CD40 mAb 89 and the control mAb A4 were used at 50 µg/ml.
The cultures were harvested 3 days later after addition of ^{3}H
Thymidine in the last 16 hours of culture. The values represent
means and standard deviations of triplicate cultures.

IL-13 Induces Ig Production by COS-7/hCD40-L Stimulated B Cells

COS-7 cells expressing human or mouse CD40-L also induced Ig production by highly purified naive surface IgD+ human B cells in the presence of IL-4 or IL-13 (Table 6). Considerable levels of IgM,

5   IgG4, total IgG and IgE, but no IgA were produced. There was no IgA production, compatible with previous observations which indicated that IL-4 specifically inhibits IgA synthesis under these culture conditions. Ig levels induced by IL-13 were in the same range as those induced by IL-4.

10   No Ig production was obtained in the presence of mock-transfected COS-7 cells (Table 6). Induction of all Ig isotypes by COS-7 cells expressing CD40-L was effectively blocked by CD40-Ig (10 μg/ml), confirming that specific engagement of the CD40-L is necessary for induction of B cell differentiation and Ig production.

15   Inhibition of total IgG production by CD40-Ig could not be measured, since the Ig portion of the CD40-Ig fusion protein gave a strong signal in the IgG ELISA.

Ig production, including IgG4 and IgE production, induced by IL-13 in the presence of COS-7/CD40-L cells was not blocked by

20   anti-IL-4 mAbs (10 μg/ml), whereas these mAbs strongly blocked IL-4-induced Ig production in the presence of COS-7/CD40-L (Table 6). These results indicate that IL-13 induces Ig production independently from IL-4. They also indicate that IL-13 is another cytokine that directs naive surface IgD+ human B cells to switch to

25   IgG4 and IgE producing cells in the presence of a contact-mediated costimulatory signal delivered by COS-7 cells expressing the mouse or human CD40-L.

30

Table 6: Induction of Ig Synthesis by IL-13 or IL-4 and COS Cells Expressing CD40-L

	IgM	IgG	IgG4	IgE
			(ng/ml)	
COS hCD40-L	4 ± 2	38 ± 4	12 ± 1	<0.2
COS mCD40-L	<0.2	6 ± 0	3 ± 0	<0.2
IL-4+COS hCD40-L	87 ± 8	195 ± 21	148 ± 30	80 ± 4
IL-4+COS hCD40-L+CD40-Ig	3 ± 1	ND*	1.8 ± 1.4	2.7 ± 1
IL-4+COS hCD40-L+anti-IL-4	8 ± 3	28 ± 7	5 ± 3	4 ± 2
IL-4+COS mCD40-L	64 ± 6	208 ± 5	177 ± 42	68 ± 7
IL-4+COS mCD40-L+CD40-Ig	<0.2	ND*	<0.4	<0.2
IL-4+COS	0 ± 0	4 ± 0	1 ± 0	<0.2
IL-13+COS hCD40-L	51 ± 1	151 ± 9	127 ± 9	54 ± 7
IL-13+COS mCD40-L	31 ± 3	100 ± 2	55 ± 0	37 ± 6
IL-13+COS	3 ± 3	5 ± 0	1 ± 1	<0.2
IL-13+COS hCD40-L+antiIL-4	48 ± 8	167 ± 12	111 ± 7	48 ± 4
-	<0.2	8 ± 1	2 ± 0	<0.2
IL-13	<0.2	7 ± 0	1 ± 1	<0.2
IL-4	<0.2	4 ± 1	2 ± 0	<0.2

Highly purified sIgD$^+$ splenic B cells (5,000 cells/well) were co-cultured with pJFE14 vector transfected or sorted COS-7 cells (250 Cells/well) transfected with and expressing the human (h) or mouse (m) CD40-L. IL-13 (30 ng/ml) and IL-4 (400 U/ml) were added as indicated. The sensitivities of the ELISAs (0.2 ng/ml for IgE and IgM, 0.4 ng/ml for IgG4 and total IgG) were determined with calibrated Ig standards (Behring, Marburg, Germany).
*No IgG determination was possible as there was detection of the Ig portion of CD40-Ig fusion protein added.

Expression and Distribution of the hCD40-L

Resting CD4$^+$ T cell clones expressed no, or very low levels of CD40-L, as judged by binding of PE-labelled streptavidin to biotinylated CD40-Ig bound to the T cells. However, significant expression of the CD40-L was observed on the CD4$^+$ T cell clone B21 4 h after activation with PHA. Consistent with its presence on the surface of B21 cells, hCD40-L mRNA was detected by Northern

analysis and by PCR. Low levels of hCD40-L mRNA were expressed
in resting B21 cells. Kinetic studies indicated that the 2.1 kb and 1.2
kb mRNA species were already maximally expressed within 2 hours
following activation, irrespective of the mode of activation of the T
5   cells.

The expression was somewhat reduced after 4 h. Considerable
reduction in the CD40-L mRNA expression was observed 7 hours
after activation, but appreciable levels of CD40-L mRNA were still
visible 48 h after activation. Activation of the B21 cells by Ca^{2+}
10   ionophore plus PMA, Con A, anti-CD3 mAbs plus PMA, or PHA plus
PMA, did not result in major quantitative differences, or differences
in the kinetics of the hCD40-L mRNA expression, although it seems
that activation with Ca^{2+} ionophore plus PMA is slightly more
effective. Distribution of the hCD40-L was analyzed by PCR, using
15   primers complimentary to the coding region of the human CD40-L
gene. CD40-L transcripts were not present in B cells, brain, kidney,
heart, fetal liver, or fetal bone marrow, but could be readily
detected in $CD4^+$ T cell clones, $CD8^+$ T cell clones, a TCR $\gamma\delta$ T-cell
clone, purified NK cells, monocytes, fetal thymocytes, and small
20   intestine. Expression of CD40-L in the small intestine may reflect
IL-13 production by infiltrating MNC.

The human CD40-L cDNA, which was cloned and expressed in
COS-7 cells is very effective in inducing human B cell activation.
COS-7/hCD40-L induced homotypic aggregation of EBV-transformed
25   and normal B cells and B cell proliferation, similarly as observed
with anti-CD40 mAbs. In addition, differentiation of B cells into Ig
secreting plasma cells was observed in the presence of IL-4 or
IL-13. The 2.1 kb hCD40-L cDNA was isolated from a $CD8^+$ T cell
cDNA library and appeared to be a full-length clone, which was by
30   sequence comparison, identical to the 1.8 kb cDNA's described
earlier. An additional 1.2 kb cDNA clone probably represents a
second mRNA species of that size which was detected in activated T
cells and which apparently encodes the same protein.

The hCD40-L has 80% homology with the corresponding mouse
35   gene. Interestingly, the hCD40-L has also some degree of homology
with TNF-α and TNF-β. The positioning of the four cysteine residues

and the potential extracellular N-linked glycosylation site in the mouse CD40-L are conserved in the human CD40-L, however the human protein has an additional cysteine substituted at position 194. The CD40-L is reported to be a type II membrane anchored

5 protein and there is a hydrophobic region of the human protein (amino acids 22-45) representing a potential signal/anchor domain near the amino terminus. B cell proliferation induced by COS-7/CD40-L was enhanced by IL-4 or IL-13. IL-4 and IL-13 seemed to be equally effective, indicating that IL-13, like IL-4, has B cell

10 growth promoting activity. IL-13, like IL-4, also induced Ig production in cultures of naive surface IgD$^+$ B cells that have been co-stimulated by COS-7/hCD40-L.

Considerable levels of IgM, IgG4, total IgG, and IgE were produced under these culture conditions. The profile of Ig

15 production induced by IL-4 and IL-13 with hCD40-L is similar to that obtained in the presence of IL-4 and anti-CD40 mAbs. Thus IL-13 and IL-4 appear equally potent in inducing both proliferation and Ig synthesis in B cells. Furthermore, these results indicate that the hCD40-L provides a co-stimulatory signal for IL-4 or IL-13

20 induced B cell differentiation, confirming the important role for CD40 in B cell activation and differentiation. Since these experiments were carried out with naive sIgD$^+$, these results confirm previous observations that IL-13, in addition to IL-4, is another CD4$^+$ T cell derived lymphokine that can direct B cells to

25 switch to IgG4 and IgE producing cells. Ig production, including IgG4 and IgE production, induced by IL-13 in the presence of hCD40-L was not blocked by anti-IL-4 mAbs, indicating that the effects of IL-13 are mediated independently of IL-4.

Help provided by the CD40-L transfectants, and the specific

30 blocking of this help by CD40-Ig, indicated that expression of CD40-L on CD4$^+$ T cells may be an important component of both antigenic and non-specific T-B cell interactions, leading to B cell activation and differentiation. These data are compatible with blocking studies carried out with mAbs against mouse CD40-L, or CD40-Ig, which

35 indicated that CD40-L and CD40 interaction is critical for T cell help in the mouse system. It is of importance to note that there is a

difference in the consequences of signaling by CD40 and activated
CD4$^+$ T cells suggesting that additional T cell surface molecules may
be involved in productive T-B cell interaction. In fact, the
transmembrane form of TNF-α expressed on activated CD4$^+$ T cells is

5 also associated with T cell induced B cell activation and
differentiation.

Considering these similarities in functions, it is interesting that
the CD40-L and the cell surface form of TNF-α are homologous and
share some structural similarities, as do CD40 and the TNF receptor.

10 The substantial help given to human B cells by the cloned human
and mouse CD40-L's is consistent with previous studies,
demonstrating that signaling through CD40 is of significant
consequence for B cell survival, activation, and differentiation. The
mouse CD40-L appears as effective as the human CD40-L in

15 activating human B cells, which is consistent with the ability of
murine CD40-L to bind human CD40. The similarity of the protein
sequences and the ability of both mouse and human-CD40-L to bind
CD40 cross-species indicates this is an important interaction _in vivo_
to be so well conserved.

20 Human IL-13 cDNA was isolated form the same CD8$^+$ T-cell
clone library as CD40-L. However, although IL-13 is expressed in
the CD8$^+$ T cells, far more IL-13 is expressed in CD4$^+$ T cells. The
potency of the combination of these two novel molecules for
induction of IgE synthesis, and their abundant co-expression by

25 CD4$^+$ T cells, together with the prolonged expression of IL-13 mRNA
following T cell activation may be a mechanism contributing to IgE
production _in vivo_ and IgE mediated allergic reactions.

These experiments have focused on the function of CD40-L
expressed on CD4$^+$ T cells as it relates to B cell activation. CD40-L

30 expression on cells other than CD4$^+$ T cells, including CD8$^+$ T cells
from which the gene was cloned, suggests a broader function for the
molecule than in T-B cell interaction. It is likely that CD40-L is
expressed on other cell types, and even CD4$^+$ T cells, which will have
important consequences for their function, rather than just

35 providing a one way stimulus to CD40 positive cells such as B cells.
For example CD40-L and CD40 expression, in thymocytes and thymic

epithelium may be indicative of interactions involved in T cell
development.

    E. Igε Switching

    The present series of experiments demonstrated that IL-13
5   induced IgG4 and IgE synthesis by human B cells. IL-13 induced
    IgG4 and IgE synthesis by unfractionated peripheral blood
    mononuclear cells (PBMNC) and highly purified B cells cultured in
    the presence of activated $CD4^+$ T cells or their membranes. IL-13-
    induced IgG4 and IgE synthesis was IL-4-independent, since it was
10 not affected by neutralizing anti-IL-4 monoclonal antibody (mAb).
    Highly purified $sIgD^+$ B cells could also be induced to produce IgG4
    and IgE by IL-13, indicating that the production of these isotypes
    reflected IgG4 and IgE switching and not a selective outgrowth of
    committed B cells.   IL-4 and IL-13, added together at optimal
15 concentrations, had no additive or synergistic effect, suggesting that
    common signaling pathways may be involved.
    This notion is supported by the observation that IL-13, like
    IL-4, induced CD23 expression on B cells and enhanced CD72, surface
    IgM (sIgM), and class II MHC antigen expression. In addition, like
20 IL-4, IL-13 induced germline ε transcription in highly purified B
    cells. Collectively, these data indicated that IL-13 is another T cell-
    derived cytokine that, in addition to IL-4, efficiently directs naive
    human B cells to switch to IgG4 and IgE production.
    B cells undergo Ig isotype switching and differentiation into
25 Ig-secreting cells in response to sIgM mediated signals in the
    presence of costimulatory factors provided by $CD4^+$ T cells. Antigen-
    specific T-B cell interactions require binding of the T cell receptor to
    peptide-class II major histocompatibility complexes (MHC) on B
    cells, which results in T-cell activation and cytokine synthesis. Once
30 the T cells are activated they can activate B cells in an antigen-
    independent fashion.
    Cytokines are essential for B-cell proliferation and
    differentiation; they not only determine Ig secretion quantitatively,
    but they also direct Ig isotype switching. IL-4 induces IgG4 and IgE

switching, whereas transforming growth factor-β (TGF-β) directs IgA
switching. See, e.g., Van Vlasselaer *et al.*, *J. Immunol. 148*:2062
(1992); and Defrance *et al.*, *J. Exp. Med. 175*:671 (1992). In addition
to cytokines, contact-mediated signals delivered by CD4^{+} T cells are
required for B cell proliferation and Ig production. Above, the
ligand for CD40, which is expressed on activated CD4^{+} T cells, was
shown to be one such membrane associated molecule that acts as a
costimulatory signal for IL-4-dependent IgE production by both
murine and human B cells. See, e.g., Armitage *et al.*, *Nature 357*:80
(1992). Moreover, several cytokines, such as IL-2, IL-5, IL-6, IL-8,
IL-10, IL-12, interferon-α (IFN-α), IFN-γ, tumor necrosis factor-α
(TNF-α), and TGF-β modulate IL-4-induced IgG4 and IgE synthesis.

    IL-4 has been thought to be the only cytokine capable of
inducing IgE synthesis. Out of 16 cytokines tested, IL-4 was the
only one inducing germline or productive ε transcripts or IgE
synthesis. In addition, anti-IL-4 mAbs preferentially inhibit IgE
synthesis induced by IL-4 producing T cell clones without
significantly affecting IgM, IgG, or IgA synthesis. Also in murine
models, anti-IL-4 antibodies strongly inhibit IgE synthesis *in vivo*
without affecting the other Ig isotypes. Most importantly, IL-4
deficient mice lack IgE in their sera following nematode infection.
However, a non-IL-4-producing T cell clone induces germline ε
transcription in purified B cells indicating that an IL-4-independent
pathway of induction of germline ε transcription is operational.
IL-13 induces CD23 expression and germline ε mRNA synthesis and
IgG4 and IgE switching in human B cells.

Reagents

    Human recombinant IL-13 was purified as described herein.
Recombinant IL-4, IFN-α, and IFN-γ were provided by Schering-
Plough Research (Bloomfield, NJ). Fluorescein isothiocyanate (FITC)-
conjugated anti-CD72 mAb and neutralizing anti-TGF-β mAb were
purchased from R&D Systems, Inc. (Minneapolis, MN). FITC- and
phycoerythrin (PE)-conjugated mAbs specific for CD3, CD4, CD8,
CD14, CD16, CD19, CD20, CD23, CD25, CD56, HLA-DR, and control

antibodies with irrelevant specificities were obtained from Becton-Dickinson (Mountain View, CA). FITC- or PE-conjugated mAbs specific for LFA-1 (L130), LFA-3, ICAM-1 (LB2), B7 (L307), and class I MHC antigen were kindly provided by Dr. J. Phillips (DNAX).

5 FITC-conjugated anti-IgD and anti-IgM mAbs were obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). The purified anti-CD40 mAb 89 (IgG1) described by Banchereau *et al.*, *Science 251*:70 (1991), was a gift of Dr. J. Banchereau (Schering-Plough France, Dardilly, France). The neutralizing anti-IL-4 mAb

10 25D2.11 was kindly provided by Dr. J. Abrams (DNAX).

Cell Preparations

Blood samples and spleens were obtained from healthy volunteers or from patients undergoing splenectomy due to trauma,

15 respectively. Mononuclear cells were isolated by centrifugation over Histopaque-1077 (Sigma, St. Louis, MO).

Purified B cells were obtained by negative sorting using a fluorescence-activated cell sorter FACStar Plus (Becton-Dickinson) or magnetic beads (Dakopatts, Norway). Briefly, splenic MNC were

20 washed twice and PE-conjugated mAbs against CD3, CD4, CD8, CD14, CD16, and CD56 were added at saturating concentrations and incubated at 4°C for 30 min. The cells were washed twice with PBS. Cells with the light scatter characteristics of lymphocytes were gated, and PE⁻ cells were sorted. Alternatively, cells stained with

25 mAbs against CD3, CD4, CD8, CD14, CD16, and CD56 were incubated for 30 min at 4°C with magnetic beads coated with anti-mouse Ig mAbs.

The cells bearing murine Ig were removed using a magnetic field. The remaining cells were washed, counted, and used in

30 further experiments. For isolation of sIgD⁺ B cells, positive sorting by FACStar Plus was used. Splenic MNC were stained with PE-conjugated mAbs against CD3, CD4, CD8, CD14, CD16, and CD56 and FITC-conjugated anti-IgD mAb, and FITC⁺, PE⁻ cells were sorted. On reanalysis, purities of the sorted cell populations were >98%, and

35 that of cells isolated using magnetic beads >95%.

The CD4+ T cell clone B21 and the CD4+ non-IL-4 producing T
cell clone SP-A3 were cultured according to Roncarolo *et al., J. Exp.
Med. 167*:1523 (1988). The cells were obtained 4-6 days after they
had been activated by the feeder cell mixture and PHA. In addition,
5   IL-2 (100 U/ml) was added to maintain the activation state of the T
cell clones.

Preparation of T Cell Membranes

The membranes of a CD4+ T cell clone were prepared according
10   to Gascan *et al., Eur. J. Immunol. 22*:1133 (1992). Briefly, the CD4+ T
cell clone B21 was harvested 12 days after activation with feeder
cell mixture and phytohemagglutinin (PHA), the cells were washed
and restimulated with 10 μg/ml of Concanavalin A (Con A) for 7-8 h
at 37° C. During the last 30 min of the Con A stimulation, 100 μg/ml
15   of α-methyl-D-mannoside (Sigma, St. Louis, MO) was added. From
these cells, membranes were prepared using the method described
by Brian, *Proc. Natl. Acad. Sci.* USA *85*:564 (1988); and Maeda   *et
al., Biochim. Biophys. Acta 731*:115 (1983); and they were stored
under liquid nitrogen (1 x 10^8 T cell equivalents/ml = 0.2 mg
20   protein/ml membrane preparation) until used.

Culture Conditions

Purified B cells were cultured at 5000 cells/well in
quadruplicate, in round-bottomed 96-well plates (Linbro, McLean,
VA) at 37°C in a humidified atmosphere containing 5% CO_2 in 0.2ml
25   Yssel's medium supplemented with 10% fetal calf serum (FCS).
Unfractionated PBMNC were cultured at 10^5 cells/well in 12
replicates. In coculture experiments, the CD4+ T cell clone SP-A3
was cultured at 5000 cells/well (T:B cell ratio 1:1). After a culture
period of 12 days, Ig levels in the culture supernatants were
30   measured by ELISA.

Measurement of Ig Production

IgM, total IgG, IgA, and IgE secretion were determined by
ELISA as described in Pène *et al.*, *Proc. Natl. Acad. Sci.* USA *85*:6880
(1988).   IgG4 secretion was determined by ELISA as described in
5   Punnonen *et al.*, *J. Immunol. 148*:3398 (1992).   The sensitivities of
IgM, total IgG, and IgA ELISAs were 0.5-1 ng/ml, and the
sensitivities of IgG4 and IgE ELISAs were 0.2 ng/ml.

Phenotypic analysis of cultured cells.

10   Purified B cells were cultured as described above and were
harvested and washed twice.   FITC- and PE-conjugated mAbs were
added at saturating concentrations and incubated at 4°C for 30 min.
FITC- and PE-conjugated mAbs with irrelevant specificities were
used as negative controls.   The cells were washed twice with PBS
15   and cells with the light scatter characteristics of lymphocytes were
analyzed using a FACScan flow cytometer (Becton-Dickinson).

RNA Isolation and Northern Analysis

Total RNA was isolated using RNAzol B (CNNA: Biotech,
Friendswood, TX) according to manufacturer's instructions.   RNA was
20   electrophoresed through 0.85% agarose and transferred to BA-S
nitrocellulose (Schleicher and Schuell, Keone, NH).   32P cDNA probes
were made by random priming using as templates, an *Eco*RI/*Hind*III
fragment of pBSIgE1-4 for germline ε, and DNA complementary to
the *Bgl*I/*Sma*I fragment of pHFgA-1 for actin.   See Gauchat *et al.*, *J.*
25   *Exp. Med. 172*:463 (1990); and Erba *et al.*, *Nucleic Acids Res. 14*:5275
(1986).

IL-13 Induces CD23 Expression on Purified B Cells

The effect of IL-13 on the expression of a variety of B cell
surface antigens was investigated by FACS analysis.   Incubation of
30   purified B cells with IL-13 (200 U/ml) resulted in strong induction
of CD23 expression on a proportion (about 20%) of the B cells.   IL-13
also upregulated class II MHC antigen, sIgM, and CD72 expression on

B cells. These effects of IL-13 were similar to those observed by
IL-4. CD23 expression was already detectable after a culture period
of 24 h, but maximal responses were observed after 72 h of culture.
The expression of CD19, CD20, CD25, CD40, class I MHC antigen, B7,
5   ICAM-1, LFA-1, and LFA-3 were not significantly modified by IL-13.

IL-13 Induces IgE Synthesis by PBMNC.

Because CD23 expression on B cells has been associated with
IgE synthesis, IL-13 was tested for induction of IgE synthesis by
human PBMNC. As shown in Table 7, IL-13 induced IgE synthesis
10   by unfractionated PBMNC in a dose-dependent manner in the
absence of exogenous IL-4. In addition, strong IgG4 production in
response to IL-13 was observed.

Neutralizing anti-IL-4 mAbs failed to inhibit IL-13-induced
IgE synthesis, whereas IL-4-induced IgE production was virtually
15   completely blocked, indicating that IL-13-induced IgE synthesis was
not mediated through induction of IL-4 production by PBMNC. See
Table 8. Similarly to IL-4, maximal induction of IgE synthesis by
IL-13 was usually obtained at concentrations of 50 U/ml. The mean
level of IgE produced in response to IL-13 was somewhat lower (63
20   ng/ml, n=6) than that induced by IL-4 (169 ng/ml, n=6). No
additive or synergistic effects were observed when both IL-4 and
IL-13 were used at saturating concentrations.

Table 7: Induction of IgE and IgG4 Synthesis By IL-13
25

	IgE (ng/ml)	IgG4 (ng/ml)
Medium	<0.2	31±14
IL-13	62±17	413±138

30

35

Table 8:   Induction of IgE Synthesis By IL-4 and IL-13; Effect of
Anti-IL-4 mAbs

	IgE Synthesis (ng/ml)
Medium	<0.2
IL-13 (0.5 U/ml)	<0.2
IL-13 (5 U/ml)	13±3
IL-13 (50 U/ml)	35±13
IL-13 (500 U/ml)	32±10
IL-13 (500 U/ml)+anti-IL-4 mAb	26±5

	IgE Synthesis (ng/ml)
Medium	<0.2
IL-4 (0.5 U/ml)	<0.2
IL-4 (5 U/ml)	6±3
IL-4 (50 U/ml)	68±13
IL-4 (500 U/ml)	71±11
IL-4 (500 U/ml)+IL-13 (500U/ml)	75±10
IL-4 (500 U/ml)+anti-IL-4 mAb	4±1

IL-13 Induces IgG4 and IgE Switching in B Cells

The ability of IL-13 to induce IgG4 and IgE synthesis by
purified B cells was also tested. It was thereby found that IL-13
induced IgG4 and IgE synthesis by highly purified B cells cultured in
the presence of membranes of an activated CD4+ T cell clone. Also in
this culture system the levels of IL-13-induced IgG4 and IgE
production were generally lower than those induced by IL-4. The
difference was in the same range as observed in the cultures of
unfractionated PBMNC. IL-13 also induced significant levels of IgM
and total IgG production, but no IgA synthesis was observed.

In this aspect IL-13 has properties similar to IL-4, which
generally inhibits IgA synthesis. See van Vlasselaer *et al.*, *J.
Immunol.* *148*:1674 (1992). These results show that IL-13 induces

IgG4 and IgE synthesis by human B cells in the absence of IL-4, and
indicate that IL-13 acts directly on B cells to induce IgG4 and IgE
synthesis. Furthermore, these results strongly suggest that IL-13
induces Ig isotype switching to IgG4 and IgE in an IL-4-independent
5   manner.

    To confirm that IgE synthesis observed in above experiments
was due to Ig isotype switching and not to an outgrowth of a few
IgE-committed B cells, the effects of IL-13 on naive sIgD+ B cells
were studied. Culturing of highly purified sIgD+ B cells with the
10 activated, non-IL-4 producing T cell clone SP-A3 in the presence of
IL-13 resulted in induction of IgE synthesis. In addition, IL-13
enhanced IgG4 synthesis induced by this non-IL-4 producing T cell
clone alone. As was demonstrated for PBMNC, IL-13-induced IgG4
and IgE synthesis could not be inhibited by anti-IL-4 mAbs.

15 Induction of Germline ε Transcription by IL-13

    So far, IL-4 has been the only cytokine known to induce
germline ε transcription in B cells. Since switching to ε by IL-4 is
preceded by the induction of germline ε RNA synthesis, it was
hypothesized that IL-13 would induce germline ε transcription as
20 well. Indeed, when highly purified B cells were cultured in the
presence of IL-13 and anti-CD40 mAbs, germline ε mRNA synthesis,
at levels comparable to that in the presence of IL-4 and anti-CD40
mAbs, was detected after a culture period of five days (see Tables 9
and 10). Since anti-CD40 mAbs alone did not induce germline ε
25 transcription in B cells, these results indicated that IL-13 is another
T cell-derived cytokine that, like IL-4, could induce germline ε
transcripts in B cells. In addition, these results confirmed the
correlation between germline ε transcription and subsequent
switching to IgE synthesis.

30

35

Table 9: IL-13 Induces Ig Synthesis By Fetal BM Cells Cultured In The Presence of Anti-CD40 mAbs

	IgM	IgG	IgG4	IgE
Medium	<1	<1	<0.2	<0.2
anti-CD40 (10 μg/ml)	<1	<1	<0.2	<0.2
anti-CD40 (10 μg/ml) +IL-13 (400 U/ml)	5 ± 2	10 ± 3	2 ± 2	1 ± 1

Table 10: IL-13 Induces Ig Synthesis By CD19$^+$, sIgM$^+$ Immature B Cells and CD19$^+$, sIgM$^-$ Pre-B Cells

Sorted CD19$^+$, sIgM$^+$ fetal B cells:

	IgM	IgG	IgG4	IgE
Medium	<1	<1	<0.2	<0.2
B21	<1	<1	<0.2	<0.2
B21+IL-13	<1	<1	<0.2	<0.2
B21+IL-7	<1	<1	<0.2	<0.2
B21+IL-7+IL-13	8 ± 2	23 ± 6	6 ± 2	2 ± 1
B21+IL-7+IL-13 +anti-IL-4 mAb	7 ± 2	21 ± 4	2 ± 1	2 ± 1

Sorted CD19$^+$, sIgM$^+$ fetal B cells:

	IgM	IgG	IgG4	IgE
Medium	<1	<1	<0.2	<0.2
B21	<1	<1	<0.2	<0.2
B21+IL-13	<1	<1	<0.2	<0.2
B21+IL-7	<1	<1	<0.2	<0.2
B21+IL-7+IL-13	5 ± 1	6 ± 1	2 ± 1	1 ± 1
B21+IL-7+IL-13 +anti-IL-4 mAb	6 ± 1	3 ± 2	1 ± 1	2 ± 1

Effect of IL-12 on IL-13 Plus Anti-CD40-induced IgE Synthesis.

Ten thousand highly purified B cells were cultured in the presence of anti-CD40 monoclonal antibodies (20 µg/ml) and IL-13
5   (400 U/ml) or IL-4 (400 U/ml). COS supernatant containing IL-12 or mock COS supernatant was added, and IgE was measured by ELISA after 14 days of culture. IL-12 decreased the IL-13 effect, while increasing the IL-4 effect.

IL-4 has been considered the only cytokine to induce IgE
10   switching in human or murine B cells. This was based on studies showing that anti-IL-4 mAbs preferentially block IgE synthesis both *in vitro* and *in vivo*, and on the observation that no circulatory IgE could be detected in mice, in which the IL-4 gene had been disrupted. It was found, however, that IL-13-induced IgE synthesis
15   is independent of IL-4, since IL-13 induced IgG4 and IgE synthesis in cultures of highly purified B cells in the absence of exogenous IL-4. In addition, anti-IL-4 mAbs, which efficiently blocked IL-4-induced IgE synthesis, failed to affect IL-13-induced IgE production. Moreover, IL-13-induced IgG4 and IgE synthesis, like that induced
20   by IL-4, reflects Ig isotype switching and is not due to a selective outgrowth of a few B cells committed to IgG4 or IgE synthesis, since IL-13 also induced IgG4 and IgE synthesis by naive, sorted sIgD+ B cells.

Switching to IgE by IL-13 was preceded by induction of
25   germline ε mRNA synthesis, but costimulatory signals provided by activated T cells were required for induction of IgE production. This is consistent with studies showing that IL-4-induced switching to ε in both murine and human B cells is preceded by the induction of germline ε RNA synthesis, and that co-stimulatory signals provided
30   by activated CD4+ T-cell clones or anti-CD40 mAbs are required for the induction of productive ε mRNA transcripts and IgE synthesis by IL-4. Although their exact role remains to be determined, it has been suggested that germline ε transcripts play an important role in the ε-switch process.

Despite the fact that IL-4 has been considered to be the only cytokine to induce germline ε transcription in B cells, an IL-4-independent pathway of induction of germline ε transcription is operational, since a non-IL-4 producing T cell clone was also capable

5 of inducing strong germline ε RNA synthesis. It is likely that IL-13 produced by the non-IL-4 producing T cell clones is responsible for the IL-4-independent induction of germline ε mRNA in B cells. The present findings may also explain why induction of IgE synthesis by IL-4 producing T-cell clones was never completely inhibited by

10 anti-IL-4 mAbs. A combination of IL-4 and IL-13 antagonists may be quite effective in blocking the switching process, each present at lower levels, e.g., below threshold levels for adverse side effects.

No additive or synergistic effects on IgE synthesis were observed when IL-4 and IL-13 were added together at optimal

15 concentrations, suggesting that IL-4 and IL-13 may use common signaling pathways for induction of IgG4 and IgE switching. Indeed, recent studies have shown that receptors for IL-13 and IL-4 share a common subunit that functions in signal transduction. However, IL-13 did not bind to cells bearing the 130 kDa IL-4 receptor

20 indicating that IL-13 does not act through this IL-4 binding protein.

The commonality between IL-13 and IL-4 was further supported by the observation that IL-13, like IL-4, induced CD23 expression on purified B cells. Similarly to IL-4, IL-13 also upregulated expression of class II MHC antigen, sIgM, and CD72,

25 which is the ligand for CD5. Although the exact role of CD23 in the regulation of IgE synthesis remains to be determined, a strong correlation between CD23 expression and induction of IgE synthesis was observed and soluble forms of CD23 were found to enhance IgE synthesis. Since IL-13 induced significant expression of CD23 within

30 24 h, these data also indicated that CD23 expression preceded IL-13-induced ε switching, thereby confirming the correlation between induction of CD23 expression and subsequent IgE synthesis.

Despite the similarities between IL-4 and IL-13 in their effects on B cells, the functions of IL-4 and IL-13 are not identical.

35 The levels of IgG4 and IgE produced in response to IL-13 were generally lower than those induced by IL-4. Moreover, preliminary

results indicated that IL-13, in contrast to IL-4, does not act on T
cells or T-cell clones. IL-13 has no obvious T cell growth promoting
activity and appears not to induce CD8a expression on CD4^{+} T-cell
clones, which may be due to lack of functional IL-13 receptors on T

5   cells. The activation state of T cells was essential for their ability to
deliver co-stimulatory signals required for B cell proliferation and
differentiation. Therefore, the lack of T cell activation inducing
effect of IL-13 may partially explain why maximal IgG4 and IgE
synthesis by PBMNC in response to IL-13 was lower than that

10   induced by IL-4.

These data seem to be incompatible with the finding that IL-4
deficient mice have no detectable circulatory IgE following
nematode infections. However, it is not clear whether IL-13 also
induces IgE synthesis by murine B cells. Preliminary data showed

15   that IL-13 was produced for much longer periods than IL-4
following T cell activation, suggesting an important role for IL-13 in
the regulation of enhanced IgE synthesis in allergic individuals.

IV. Activities on PBMC and Macrophages

A. Induction of Morphological Change in Non-adherent Human
20   PBMC

Peripheral blood mononuclear cells (PBMNC) were isolated
from normal health human donors by centrifugation over Ficoll-
Hypaque. Total PBMNC (1 x 10^8 cells) were incubated for 30
minutes at 37°C in 10 mm tissue culture dishes. Nonadherent cells

25   were removed by extensive washing of the dish with phosphate
buffered saline (PBS). Adherent cells were incubated in Yssels's
medium Yssel *et al.*, *J. Immunol. Methods* 72:219 (1974) with 1%
human AB serum alone, or with mouse P600 derived from *E. coli* (lot
560-137-1; used at a concentration of 30 ng/ml), as described

30   above. Alternatively, COS-7 derived mouse P600 or human IL-13
was used at a final dilution of 1/20. Cells were observed at regular
intervals.

B. Modification of Cell Surface Markers on Non-adherent Cells

Five or ten days after nonadherence selection as described above, the resulting cells were analyzed for expression of cell surface markers by fluorescence activated cell sorting (FACS), e.g., as described in Shapiro, *Practical Flow Cytometry* (2nd Ed.), 1988, Alan Liss, New York. Exemplary antibodies for recognizing each marker are: CD11a [LFA-1; SFN-L7, from DNAX, Palo Alto, CA], CD11b [Bear1, see Spits *et al.*, *Eur. J. Immunol. 14*:229 (1984)], CD11c [p150; NGH 93, see Visser *et al.*, *Blood 74*:320 (1989)]; CD54 [ICAM; LB2, see Azuma *et al.*, *J. Expt'l Med. 175*:353 (1992)], Class I MHC [W6/32, from Sera Labs, see also Barnstable *et al.*, *Cell 14*:9 (1978)]; Class II MHC [Q5/13, see Quaranta *et al.*, *J. Immunol. 125*:1421 (1980)]; Class II MHC [PdV5.2, see Koning *et al.*, *Human Immunol. 9*:221 (1984)]; Class II MHC (DQ; SPV-L3), CD58 [LFA-3; TS 2/9, see Krensky *et al.*, *J. Immunol. 132*:2180 (1984)], CD32 [IV.3, see Looney *et al.*, *J. Immunol. 136*:1641 (1986)], CD16 [granulocyte-1, see Huizinga *et al.*, *Nature 333*:667 (1988)]; or Leu 11a, Becton Dickinson, Mountain View, CA); CD23 (gp25, from DNAX, Palo Alto, CA), IL-2Ra [7G7; or BB10, see Herve *et al.*, *Blood 75*:1017 (1990)], CD44 [NkI-P1; see Vennegoor *et al.*, *J. Immunol. 148*:1093 (1992)], CD14 (LeuM3, Becton Dickinson), and CD18 and B7 [L130 and L307; both described in Azuma *et al.*, *J. Immunol. 149*:1115 (1992)].

Mouse P600 material from either COS-7 supernatants or *E. coli* inclusion bodies were compared to COS-7 supernatants of human IL-13.

C. Nitric Oxide Synthesis

IL-13 (P600) was assayed by its inhibitory effect on the production of nitric oxide (NO) by GM-CSF-derived bone marrow macrophages. The macrophages were derived by 9-12 days culture in RPMI containing GM-CSF and purified by retention of adherent, GM-CSF-responsive fraction. Cells were 99+% pure, as determined by FACS analysis using two color staining.

Macrophages were activated to produce NO by stimulation with LPS at 3 µg/ml in the appropriate experiments, either with or without prior stimulation with cytokines, as indicated. The

macrophages were incubated for 16 h with the cytokines (if used) 16 h prior to treatment with LPS. Supernatants were taken at the indicated times relative to LPS addition, i.e., 0 h is the time of addition of LPS.

5   Supernatants were assayed for NO production by the standard Griess assay for nitrites. See, e.g., Coligan, *Current Protocols in Immunology*, (1991 and periodic supplements) Greene/Wiley, New York. Addition of cytokines after addition of LPS to the macrophage cultures or at the time of LPS addition has been tested; under these
10   conditions, none of the cytokines tested (including IL-13) had significant effects. Other macrophages were also tested, but since they generally produced lower levels of NO, they were not used as extensively for bioassay.

        Table 11, part A shows NO production from GM-CSF-treated
15   bone marrow derived macrophages after treatment for 16 h with designated cytokines. Note that IFN-γ induced NO production, while IL-4 or IL-13 inhibited NO production. L-NMMA is a specific inhibitor of NO production. Parts B and C are similar experiments titrated over different ranges of P600 amounts. In each case, the
20   IL-13 decreased the production of NO.

5

Table 11: NO from GM-CSF-derived Macrophages (nM)

Treatment	0 hours	24 hours	48 hours
A			
medium	1.551	3.638	3.103
+ LPS + IFN-γ	3.852	10.057	9.576
+ LPS + IL-4	1.016	1.016	3.798
+ LPS + L-NMMA	0.963	0.802	0.000
+ LPS + IL-13	0.856	0.802	0.321
+ LPS	0.963	4.708	5.189
B			
medium	0.000	0.144	0.190
LPS	0.193	4.826	8.253
LPS + L-NMMA	0.144	0.482	0.145
LPS + IFN-γ	0.917	11.872	14.189
LPS + IL-4	0.000	4.633	7.095
LPS + P600 (25 ng/ml)	0.000	2.992	4.537
LPS + P600 (50 ng/ml)	0.000	3.282	4.730
LPS + P600 (100 ng/ml)	0.261	3.137	4.488
C			
medium	0.705	2.512	2.757
LPS	0.766	2.390	2.665
LPS + IFN-γ	2.359	10.509	15.319
LPS + L-NMMA	0.643	0.735	0.950
LPS + IL-13 (400 pg/ml)	0.306	1.225	1.562
LPS + IL-13 (2 ng/ml)	0.674	1.225	1.593
LPS + IL-13 (10 ng/ml)	0.643	1.164	1.532
LPS + IL-13 (50 ng/ml)	0.613	1.072	1.409
LPS + IL-13 (250 ng/ml)	0.613	1.134	1.409

D. Modulation of IL-1α; IL-6

5   IL-13 inhibits the production of IL-1α, IL-6, IL-10, and TNFα by
     LPS activated human monocytes

         Peripheral blood mononuclear cells were isolated from normal
     healthy donors by centrifugation over Ficoll-Hypaque.   Total PBMNC
10   (100 x 10^6 cells/100 mm tissue culture dish) were incubated for 30
     min at 37° C and subsequently nonadherent cells were removed by
     extensive washing of the tissue culture dish with PBS.   Adherent
     cells were incubated in Yssels medium with 1% human AB serum in
     the absence or presence LPS (*E. coli* 0127:B8, Difco, Detroit, MI) in
15   combination with IL-4 (50 ng/ml), IL-13 (50 ng/ml), or IL-10 (100
     U/ml).   In addition, cells were activated by LPS in combination with
     IL-4 or IL-13 in the presence of neutralizing anti-IL-10 mAb 19F1
     (10 µg/ml).   Supernatants were collected after 12 hrs and
     production of IL-1α, IL-6, IL-10, and TNF-α was measured by
20   cytokine-specific ELISA.   Table 12 shows the results of these studies.

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Table 12:   Effect of IL-13 on the Production of IL-1α, IL-6, IL-10,
and TNF-α by LPS Activated Human Monocytes.

	IL-1α (ng/ml)	IL-6 (ng/ml)	IL-10 (ng/ml)	TNF-α (ng/ml)
Medium	0	0	0	0
LPS	8.1	54.4	35.4	2.2
LPS + IL-4	1.7	33.1	30	0.7
LPS + IL-13	2	35.4	22	0.5
LPS + IL-10	0	8.6	ND	0
LPS + αIL-10 mAb	12	101	ND	10.6
LPS + αIL-10 mAb + IL-4	3.7	59.5	ND	1.2
LPS + αIL-10 mAb + IL-4	5.4	79.5	ND	1.5

These results indicated that IL-4 and IL-13 inhibited the
production of IL-1α, IL-6, IL-10, and TNF-α by LPS activated human
monocytes.   IL-10 also inhibited the production of IL-1α, IL-6, and
TNF-α by LPS activated human monocytes.   IL-10 was produced by
human monocytes and inhibited IL-1α, IL-6, and TNF-α in an
autoregulatory fashion.   Addition of IL-10 neutralizing mAb 19F1
showed that endogenously produced IL-10 also inhibited the
production   of IL-1α, IL-6, and TNF-α. The inhibitory effects of
IL-4 and IL-13 on cytokine production by LPS activated human
monocytes were independent of IL-10 since IL-4 and IL-13
inhibited the production of IL-1α, IL-6, and TNF-α in the presence of
neutralizing anti-IL-10 mAb 19F1.

E. Antibody Dependent Cell-mediated Cytotoxicity (ADCC)

The present experiments investigate the effects of IL-13 alone
or in combination with IL-4, IFN-γ, or IL-10 on human monocytes.
IL-13 induced significant changes in the phenotype of monocytes.
Like IL-4, it enhanced the expression of CD11b, CD11c, CD18, CD29,
CD49e (VLA-5), class II MHC, CD13, and CD23 whereas it decreased

the expression of CD64, CD32, CD16, and CD14 in a dose-dependent manner. IL-13 induced upregulation of class II MHC antigens and its downregulatory effects on CD64, CD32, and CD16 expression were prevented by IL-10. IFN-γ could also partially prevent the IL-13 induced downregulation of CD64, but not that of CD32 and CD16. However, IL-13 strongly inhibited spontaneous and IL-10 or IFN-γ induced antibody dependent cell-mediated cytotoxicity (ADCC) activity of human monocytes toward anti-IgD coated Rh$^+$ erythrocytes, indicating that the cytotoxic activity of monocytes was inhibited.

Furthermore, IL-13 inhibited production of IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12 P35, IL-12 P40, GM-CSF, G-CSF, IFN-α and TNF-α by monocytes activated with LPS. In contrast, IL-13 enhanced the production of IL-1RA by these cells. Similar results on cytokine production were observed or have been obtained for IL-4. Thus IL-13 shares most of its activities on human monocytes with IL-4, but no additive or synergistic effects of IL-4 and IL-13 on human monocytes were observed suggesting that these cytokines may share common receptor components. Taken together, these results indicate that IL-13 has anti-inflammatory and immunoregulatory activities.

Activated T cells secrete a number of biologically active polypeptides, which regulate the proliferation, differentiation and function of cells participating in immune responses against antigens. T cells producing IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IFN-γ, GM-CSF, and TNF/LT simultaneously following antigenic or polyclonal stimulation have been described in both mouse and man. These T helper cells were designated Th0 cells in order to distinguish them from the more specialized Th1 and Th2 subsets. Murine Th1 cells produce IL-2, IFN-γ, TNF/LT, IL-3, and GM-CSF which supports their function as regulatory and effector cells in cellular immune responses such as delayed type hypersensitivity (DTH) whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, IL-3, and GM-CSF which makes them suitable for providing help to B cells in the production of immunoglobulins of different isotypes.

In man, T cell clones with restricted cytokine production profiles have also been isolated from patients with inflammatory or allergic diseases. Although these types of clones resembled murine Th1 and Th2 clones, there were some differences. Depending on their mode of activation, Th1 clones generally could still produce low quantities of IL-4 whereas Th2 clones were able to produce low to normal quantities of IFN-γ. However, a clear imbalance in the production ratios of IL-4 and IFN-γ by Th2 clones was observed following antigenic stimulation. Therefore human T cell clones were defined which produce high levels of IFN-γ and no, or low levels of IL-4 Th1 "like" cells and T cell clones which produce no, or low levels of IFN-γ and high levels of IL-4 Th2 "like" cells. Furthermore, IL-10 which is exclusively produced by Th0 and Th2 T cell subsets in the mouse, is produced by Th0, Th1 "like", and Th2 "like" subsets in man.

The present invention makes available a new cytokine, human IL-13, which is related to the mouse P600 protein. Both human IL-13 and mouse P600 proteins were biologically active and affected human monocyte and B cell functions.

The biological activities of mouse and human IL-13 on human monocytes were further characterized and compared to those of IL-4, IL-10, and IFN-γ, other cytokines with stimulatory of inhibitory actions on human monocytes. IL-13 induced dramatic changes in the phenotype of human monocytes and inhibited the production of IL-1α, IL-1β, IL-6, IL-8, IL-10, GM-CSF, G-CSF, and TNF-α following activation by LPS, whereas it induced the production of IL-1RA. These results indicate that IL-13 has anti-inflammatory activities and may play an important regulatory role in immune responses.

Isolation and Culture of Human Monocytes

Human monocytes were isolated from peripheral blood of healthy donors by centrifugation over Ficolll-Hypaque and adherence to plastic. Briefly, 100 x 10⁶ PBMNC were plated on a 100 mm tissue culture dish in Yssel's medium supplemented with

human serum albumin (HSA) and 1% pooled human AB$^+$ serum and incubated at 37° C for 30 min. This culture medium was endotoxin free as determined by the Limulus amoebocyte lysate assay (< 0.2 ng/ml of endotoxin). Subsequently, nonadherent cells were

5   removed by extensive washing and cultured in Yssel's medium with HSA and 1% pooled human AB serum as indicated. Alternatively, highly purified human peripheral blood monocytes were obtained from 500 ml blood of normal donors by centrifugal elutriation.

Mononuclear cells were isolated by density centrifugation in a

10   blood component separator, followed by fractionation into lymphocytes and monocytes. The monocyte preparation was > 95% pure, as judged by nonspecific esterase staining and contained more than 98% viable cells. These monocytes were cultured in Yssel's medium with HSA and 1% pooled human AB$^+$ serum at a

15   concentration of 4 x 10^6 cells/ml in teflon bags (Jansen MNL, St Niklaas, Belgium), which prevented adhesion of these cells. After culture for the times indicated, monocytes were collected and analyzed for cell surface expression by indirect immunofluorescence or analyzed for lymphokine gene expression by Northern and PCR

20   analysis. In addition, monocyte culture supernatants were collected for determination of IL-1α, IL-1β, IL-6, IL-8, IL-10, TNF-α, GM-CSF, G-CSF, and IL-1RA production following activation of these cells by LPS (*E. coli* 0127:B8) (Difco, Detroit, MI) at 1 μg/ml. The viability of the cells after culture always exceeded 95% as determined by

25   trypan blue exclusion.

Reagents

Recombinant human and mouse IL-13 were expressed in *E. coli* as insoluble aggregates of glutathione-S-transferase fusion proteins, extracted by centrifugation, solubilized, and subjected to

30   renaturation prior to digestion with thrombin to remove the N-terminal fusion part. Subsequently, proteins were purified by cation exchange and gel filtration chromatography, which resulted in active human and mouse IL-13. Purified human r-IL-10, r-IL-4, and r-

IFN-γ were provided by Schering-Plough Research Institute (Bloomfield, NJ).

     The neutralizing anti-IL-4 mAb 25D2 [Chretien *et al., J. Immunol. Methods, 117*:67 (1989)], and anti-IL-10 mAb 19F1

5 [Abrams *et al., Immunol. Rev. 125*:5 (1992)], were described previously. The following mAbs were used for immunofluorescence studies on the expression of cell surface markers: SPV-L7 [CD11a; Spits *et al., Hybridoma* 2:423 (1983)], Bear-1 [CD11b; Keizer *et al., Eur. J. Immunol. 15*:1142 (1985)], CLB FcR gran-1 [CD16; Klaassen *et*

10 *al., J. Immunol. 144*:599 (1990)], gp25 [CD23; Bonnefoy *et al., J. Immunol. 138*:2970 (1987)], IV.3 [CD32; Looney *et al., J. Immunol. 136*:1641 (1986)], 32.2 [CD64; Anderson *et al., J. Biol. Chem. 261*:12856 (1986)], Q5/13 [HLA-DR/DP; Quaranta *et al., J. Immunol. 125*:1421 (1980)], PdV5.2 [HLA-DR/DP/DQ; Koning *et al., Hum.*

15 *Immunol.* 9:221 (1984)], SAM-1 [VLA-5, CD49e; Keizer *et al., Eur. J. Immunol. 17*:1317 (1987)], CD29 (Ts2/16; a kind gift of C. Figdor, Amsterdam), L307 [B7; Azuma *et al., J. Immunol. 149*:1115 (1992)], IOM13 (CD13; purchased from AMAC, Inc., Westbrook, ME); Leu-M3 (CD14); Leu15 (CD11c), and L130 (CD18) were obtained from Becton-

20 Dickinson (San Jose, CA).

<u>Probes</u>

     Oligonucleotides used for Southern analysis of IL-1α, IL-1β, IL-6, IL-8, IL-10, TNF-α, GM-CSF, G-CSF, and β-actin PCR products have been described by de Waal Malefyt *et al., J. Exp. Med.*

25 *174*:1209 (1991).

     The following oligonuclotides were used to detect

IFN-α: 5'-TTCTGGCTGTGAGGAAATACT-3' (nt 360-378),

IL-1RA: 5'-GTCAATTTAGAAGAAAAGATAGATGTGG-3' (nt 207-234),

IL-12 P35: 5'-AATGGGAGTTGCCTGGCCTC-3' (nt 488-507),

30 IL-12 P40: 5'-TAAGACCTTTCTAAGATGCGAGGCC-3' (nt 417-441), and

TGF-β1: 5'-CGAGCCTGAGGCCGACTACTACGCCAAGGAGGTCACC-CGC-3'
(nt   1131-1170).

mRNA isolation and Northern analysis

Total RNA was isolated from 20 x 10^6 monocytes by a
5   guanidinium thiocyanate-CsCl procedure.   For northern analysis,
10 μg total RNA per sample was separated according to size on 1%
agarose gels containing 6.6% formaldehyde, transferred to Nytran
nylon membranes (Schleicher & Schuell, Keene, NH), and hybridized
with probes, labelled to high specific activity (> 10^8 cpm/mg) by a
10   hexamer labelling technique.   Filters were hybridized, washed under
stringent conditions, and developed.

PCR Analysis

One microgram of total RNA was reverse transcribed using
oligo (dT)$_{12-18}$ as primer (Boehringer Mannheim, Indianapolis, IN)
15   and AMV reverse transcriptase (Boehringer Mannheim) in a 20 μl
reaction volume.   Two microliters of reverse transcript (equivalent
to 100 ng of total RNA) was used directly for each amplification
reaction.   Conditions for PCR were as follows: in a 50 μl reaction
volume, 25 nmol of each primer, 125 μM each of dGTP, dATP, dCTP,
20   and dTTP (Pharmacia, Uppsala, Sweden), 50 mM KCl, 10 mM Tris-
HCl, pH 8.3, 1.5 mM MgCl$_2$, 1 mg/ml gelatin, 100 μg/ml non-
acetylated BSA, and 1 unit Vent DNA polymerase (New England
Biolabs, Beverly, MA).

Primers used to amplify IL-1α, IL-1β, IL-6, IL-8, IL-10, TNF-α,
25   GM-CSF, G-CSF, and β-actin have been described previously by de
Waal Malefyt *et al., J. Exp. Med. 174*:1209 (1991).   The following
primers were also used: IFN-α sense primer: 5'-
GCTGAAACCATCCCTGTC-3' (nt 161-178), IFN-α antisense primer:
5'-CTGCTCTGACAACCTCCCAG-3' (nt 450-430), IL-1RA sense primer:
30   5'-GCAAGCCTTCAGAATCTGGGATG-3' (nt 118-141), IL-1RA antisense
primer: 5'-GATGTTAACTGCCTCCAGCTGGAGTC-3' (nt 344-319), IL-12
P35 sense primer: 5'-CTTCACCACTCCCAAAACCTG-3' (nt 281-302),
IL-12 P35 antisense primer: 5'-AGCTCGTCACTCTGTCAATAG-3' (nt
813-792), IL-12 P40 sense primer: 5'-CATTCGCTCCTGCTGCTTCAC-3'

(nt 337-358), IL-12 P40 antisense primer: 5'-
TACTCCTTGTTGTCCCCTCTG-3' (nt 603-582), TGF-β1 sense primer
5'-ACCGGGTGGCCGGGGAGAGTGC-3' (nt 1097-1118), TGF-β1 antisense
primer: 5'-GCCGGTTGCTGAGGTATCGCCAGG-3' (nt 1399-1376).

5   Reactions were incubated in a Perkin-Elmer/Cetus DNA
Thermal cycler 9600 for 25 cycles (denaturation 30 s at 94°C,
annealing 30 s at 55°C, extension 60 s at 72°C). Forty microliter of
each reaction was loaded on 1% agarose gels in TAE buffer and PCR
products were visualized by ethidium bromide staining.

10   Subsequently, gels were denatured in 0.5 M NaOH, 1.5 M NaCl,
neutralized in 1 M ammonium acetate, and transferred to Nytran
nylon membranes. Membranes were pre-hybridized in 6 x SSC, 1%
SDS, 10 x Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone,
0.2% BSA, pentax fraction V), and 200 μg/ml E. coli tRNA

15   (Boehringer, Mannheim, FRG) for 4 h at 55°C.

        Oligonucleotide probes (200 ng), specific for a sequence
internal to the primers used in the amplification, were labelled at
the 5' end by T4 polynucleotide kinase (New England Biolabs) and
γ-^{32}P-ATP (Amersham, Arlington Heights, IL). Probes were

20   separated from non-incorporated nucleotides by passage over a Nick
column (Pharmacia, Uppsala, Sweden) and added to the
hybridization mix. Following hybridization for 12 hrs at 55°C,
filters were washed in 0.1 x SSC (1 x SSC: 150 mM NaCl, 15 mM Na-
citrate pH = 7.0), 1% SDS at room temperature, and exposed to Kodak

25   XAR-5 films for 1-2 hrs. In addition, signals were quantified on a
Molecular Dynamics phosphor-imager (Molecular Dynamics,
Sunnyvale,CA)

<u>Lymphokine Determinations</u>

        The production of cytokines by monocytes was determined in
30   culture supernatants by cytokine specific ELISA's. The cytokine
specific ELISA's and their sensitivities were the following: IL-1α,
Endogen (Boston, MA) (50 pg/ml); TNF-α, Endogen (Boston, MA)
(10 pg/ml); IL-1β, Cistron (Pine Brook, NJ) (20 pg/ml); IL-6,
Genzyme (Boston, MA) (0.313 ng/ml); IL-8, R&D Systems

(Minneapolis, MN) (4.7 pg/ml); G-CSF, R&D Systems (Minneapolis, MN) (7.2 pg/ml); IL-1RA, R&D Systems (Minneapolis, MN) (12.5 pg/ml); GM-CSF, Bacchetta *et al., J. Immunol. 144*:902 (1990), (50 pg/ml); and IL-10 (75 pg/ml).

5   Immunofluorescence Analysis

Cells (10^5) were incubated in V bottom microtiter plates (Flow Laboratories, McLean, VA) with 10 µl of purified mAb (1 mg/ml) for 30 min at 4° C. After two washes with PBS containing 0.02 mM sodium azide and 1% BSA (Sigma, St Louis, MO), the cells were
10   incubated with 1/40 dilution of FITC labelled F(ab')2 fragments of goat anti-mouse antibody (TAGO, Inc. Burlingame, CA) for 30 min at 4° C. After three additional washes, the labelled cell samples were analyzed by flow microfluometry on a FACScan (Becton Dickinson, Sunnyvale, CA.).

15   Antibody Dependent Cell-mediated Cytotoxicity (ADCC)

ADCC activity of cultured human monocytes against antibody coated rhesus positive human erythrocytes was performed as previously described by de Velde *et al., J. Immunol. 149*:4048 (1992).

20   IL-13 and IL-4 Induce Identical Changes in the Expression of Cell Surface Antigens by Human Monocytes

Both mouse and human IL-13 induced expression of CD23 (FceRII) and upregulated the expression of class II MHC antigens on human monocytes. The effects of IL-13 on the expression of a
25   larger panel of cell surface antigens was investigated. IL-13 affected the expression of multiple cell surface molecules belonging to different supergene families. IL-13 enhanced the expression of several members of the integrin superfamily of adhesion molecules. The expression of α subunits CD11b (C3bi receptor, Mac-1), CD11c
30   (gp150,95), and VLA-5 (FNR), as well as their respective β subunits CD18 (β2) and CD29 (β1, VLA-b) were upregulated by IL-13. The expression of other members of this family, including CD11a (LFA-1),

VLA-2 (CD49b), VLA-3, VLA-4 (CD49d), VLA-6 (CD49f), β3 (CD61), and β4 was not significantly affected by IL-13.

    IL-13 enhanced the expression of class II MHC antigens. The expression of HLA-DR, HLA-DP and HLA-DQ was upregulated by

5   IL-13. Expression of other members of the Immunoglobulin superfamily including class I MHC, CD11a (LFA-1), CD54 (ICAM-1), ICAM-2, and CD58 (LFA-3) was not dramatically affected by IL-13.

    IL-13 modulated the expression of the various Fc receptors on monocytes. The expression of CD64 (FcγRI), CD32 (FcγRII), and CD16

10   (FcγRIII) on human monocytes was strongly downregulated by IL-13. In contrast, IL-13 induced the expression of CD23 (FcεRII). In addition, IL-13 upregulated the expression of CD13 (Aminopeptidase N) and downregulated the expression of CD14. No major effect of IL-13 was detected on the expression of CD25,

15   CD33, and CD44.

    IL-4 induced upregulation of CD11b, CD11c, CD18, VLA-5, CD29, class II MHC, CD13, and CD23, and inhibited the expression of CD16, CD32, CD64, and CD14 on human monocytes to the same extent as did IL-13. Taken together, these results indicated that the IL-13

20   induced changes in the expression of cell surface molecules were similar to those induced by IL-4. Incubation of monocytes with saturating concentrations of both IL-4 and IL-13 did not result in changes in the phenotype as compared to those induced by either cytokine alone.

25   No additive or synergistic activities of IL-13 and IL-4 on the expression of the various cell surface molecules were detected under these conditions. There is no evidence that monocytes are able to produce IL-4. However, to exclude the possibility that IL-13 acted through the induction of IL-4 by monocytes or by a few

30   contaminating T cells, monocytes were incubated in the presence of IL-13 and a neutralizing anti-IL-4 mAb.

    As shown in Table 13, the induction of CD23, downregulation of CD14, and upregulation of class II MHC by IL-13 was not affected by the anti-IL-4 mAb. The anti-IL-4 mAb, however, was effective

35   since it completely inhibited the effects of IL-4 in control experiments. Thus, IL-13 acts independently of IL-4.

Table 13:   IL-13 Acts Independently of IL-4.

mAb	Medium	IL-13	IL-13 + αIL-4	IL-4	IL-4 + αIL-4
control	3*	5	6	5	3
MHC class II	443	1904	1845	2084	220
CD23	3	99	79	89	8
CD14	222	97	83	80	444

5 Monocytes were incubated with medium, IL-13 (50 ng/ml) or IL-4 (400 U/ml) in the absence or presence of neutralizing anti-IL-4 mAb 25D2 (10 μg/ml) at 37° C for 120 h and expression of HLA-DR/DP (Q5/13), CD23 (gp25), and CD14 (Leu-M3) was determined by indirect immunofluorescence.

10 *   Mean Fluorescence Intensity (channel number)

IL-13 induced changes in expression of cell surface markers were dose-dependent as shown for the modulation of CD11b, CD18, CD16, CD32, CD64, CD23, class II MHC, CD13, and CD14 expression

15 (Table 14).   Generally, incubation of human monocytes with 5 pg/ml⁻ IL-13 was insufficient to induce changes in the expression of these cell surface markers, whereas 0.5 ng/ml IL-13 resulted in significant changes in phenotype, comparable to those induced by 0.5 ng/ml IL-4.   Maximal responses were induced by 50 ng/ml

20 IL-13, which were again in the same range as those induced by 50 ng/ml of IL-4, indicating that IL-4 and IL-13 were equally effective.

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Table 14: IL-13 Induces Changes in Cell Surface Phenotype of
Monocytes in a Dose Dependent Manner.

mAB	IL-13 (pg/ml)				IL-4 (U/ml)	
	0	5	500	50000	4	400
control	3*	3	3	4	3	3
CD11b	59	54	102	139	102	168
CD18	71	54	79	108	99	127
CD16	25	20	20	15	20	13
CD32	50	48	44	40	43	39
CD64	57	50	36	26	31	17
CD23	4	4	12	56	7	67
MHC class II	355	386	586	607	609	908
CD13	26	26	113	121	57	102
CD14	110	110	75	37	86	16

Monocytes were incubated with medium, IL-13 (5 pg/ml, 500
pg/ml, or 50,000 pg/ml) or IL-4 (4 U/ml, or 400 U/ml) at 37°C for
120 h and the expression of cell surface antigens was determined by
indirect immunofluorescence.

*   Mean Fluorescence Intensity (channel number)

IL-10 Downregulates IL-13 Induced Class II MHC Expression on
Human Monocytes

To compare the effects of IL-13 with those of other cytokines
which modulate the cell surface phenotype, monocytes were
incubated with IL-10 or IFN-γ in the   absence or presence of IL-13
and the expression of cell surface antigens was analyzed. IL-10 or
IFN-γ alone did not dramatically affect the expression of CD11b,
CD11c, CD18, CD13, CD23, CD29, and VLA-5. In addition, IL-10 or
IFN-γ did not noticeably affect the IL-13 induced increase in
expression of these markers. IL-10 or IFN-γ had also no observed
effect on the expression of CD14 and the IL-13 induced inhibition of
CD14 expression.

However, IL-10 downregulated not only the constitutive class II expression on monocytes, but also inhibited strongly the IL-13 induced class II MHC expression. Similar data were obtained when highly purified monocytes isolated by elutriation and cultured in teflon bags were used (Table 15) Increased expression of class II MHC antigens was observed following incubation of monocytes in medium alone, which was completely prevented by IL-10. m-IL-13, h-IL-13, IL-4, and IFN-γ all induced high levels of class II MHC expression which were blocked by IL-10 (Table 15). Class II MHC expression induced by IFN-γ was further enhanced by IL-13. IFN-γ slightly upregulated expression of B7. Taken together, these results indicate that IL-13, IL-10, and IFN-γ independently modulate the expression of cell monocyte surface antigens.

Table 15: IL-10 Inhibits Constitutive and IL-13, IL-4, and IFN-γ Induced MHC Class II Expression on Human Monocytes.

Incubation	IL-10 (200 U/ml)	
	+	-
control 4°C	69*	nd**
medium 37°C	150	46
mIL-13	212	73
hIL-13	197	81
IL-4	407	94
IFN-γ	347	36

Elutriated monocytes were incubated in medium at 4°C or 37°C, mIL-13 (50 ng/ml), hIL-13 (50 ng/ml), IL-4 (400 U/ml) or IFN-γ (100 U/ml) in the absence or presence of IL-10 (200 U/ml) in teflon bags for 48 h and expression of HLA-DR/DP was determined by indirect immunoflourescence.

* Mean Fluorescence Intensity (channel number)

** Not done

IL-13 Inhibits Monocyte FcγR Cell Surface Expression and Cytotoxicity

IFN-γ, IL-4, and IL-10 are able to modulate the expression of FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) on human
5   monocytes. IFN-γ and IL-10 enhanced the expression of CD64 whereas IL-4 downregulates the expression of CD64, CD32, and CD16. Adding combinations of these cytokines to monocytes showed that IL-10 was able to prevent the IL-4 induced downregulation in cell surface expression of all three FcγR and that IFN-γ partially
10   restored the downregulatory effects of IL-4 on CD64 expression. IL-10 prevented IL-13 induced downregulation of CD64, CD32, and CD16. In addition, IFN-γ could partially rescue IL-13 induced downregulation of CD64, but did not affect the IL-13 induced downregulation of CD32 and CD16.
15   The level of ADCC activity of human monocytes has been shown to correlate with the expression of FcγRI. The effects of IL-13 on the functional activity of FcγRI on monocytes was determined by their ability to lyse anti-D opsonized human Rh^+ erythrocytes. Both human and mouse IL-13 were able to inhibit ADCC activity of
20   monocytes cultured in medium alone. On the other hand, ADCC activity was enhanced when monocytes were cultured in the presence of IFN-γ or IL-10. IL-13 significantly inhibited these effects of IFN-γ and IL-10 despite the fact that IFN-γ and IL-10 partially or completely reversed the inhibition of FcγRI expression,
25   indicating that   IL-13 affected the FcγR mediated cytotoxicity also by other mechanisms.

IL-13 Inhibits Production of Pro-inflammatory Cytokines and Hemopoietic Growth Factors But Induces IL-1RA.

To determine the effects of IL-13 on the production of
30   cytokines by human monocytes, monocytes were activated by LPS and cytokine production was determined in the culture supernatants after 6 and 24 hours by cytokine specific ELISA's. Activation of monocytes by LPS resulted in the production of IL-1α, IL-1β, IL-6, IL-8, IL-10, GM-CSF, G-CSF, TNF-α, and IL-1RA. Significant levels of

IL-1α, IL-1β, IL-6, IL-8, TNF-α, and IL-1RA were present at 6 h after activation, whereas the production of IL-10, G-CSF, and GM-CSF was detected at 24 h. At 6 and 24 h after activation, IL-13, IL-4, and IL-10 inhibited the production of IL-1α, IL-1β, IL-6, IL-8,
5   IL-10, TNF-α, G-CSF, and GM-CSF, but enhanced the production of IL-1RA.

IL-13 affected the morphology, phenotype, function, and cytokine production of monocytes. Incubation of monocytes with IL-13 induced strong adherence of these cells to plastic substrates
10   and their morphology changed to a dendritic appearance. In addition, homotypic aggregates of cells were observed. The finding that IL-13 upregulated the expression of CD11b, CD11c, CD18, VLA-5, and CD29, which are members of the integrin superfamiliy, is compatible with the observed aggregation and changes in
15   morphology, since CD11b/CD18 and CD11c/CD18 heterodimers are involved in cell-cell interactions, homotypic aggregation, adhesion to artificial substrates, and bind fibrinogen.

In addition, the α5β1 integrin VLA-5/CD29 is the receptor for fibronectin, which is an abundant extracellular matrix protein
20   involved in adhesion processes. IL-13 did not induce changes in the expression of other molecules involved in adhesion or cell-cell interaction, e.g., CD11a, VLA-2, VLA3, VLA-4, VLA-6, β3, β4, ICAM-1, ICAM-2, LFA-3, MEL-14, and CD44 but it remains possible that other cell surface structures are involved in the IL-13 induced
25   changes in morphology and adherence.

IL-13 upregulated the expression of class II MHC antigens on human monocytes. The expression of HLA-DR, HLA-DP, and HLA-DQ was significantly increased by IL-13. IL-10 inhibited constitutive and IL-4 and IFN-γ induced class II MHC expression on human
30   monocytes. IL-10 thus inhibits IL-13 induced class II MHC expression, which further supports the general immunosuppressive activities of IL-10.

The expression of the various Fc receptors for IgG and IgE on monocytes was influenced by several cytokines. CD64 (FcγRI)
35   expression was upregulated by IFN-γ and IL-10 and inhibited by IL-4. Furthermore, IFN-γ and IL-10 were able to prevent the

downregulation of CD64 induced by IL-4. Here it was shown that IL-13 inhibited the constitutive expression of CD64 and that this inhibition could also be prevented by IL-10 and IFN-γ. The expression of CD64 has been shown to correlate with ADCC activity

5 of monocytes.

Spontaneous or IL-10- or IFN-γ-induced FcγRI mediated cytotoxicity of monocytes towards IgD coated rhesus positive erythrocytes was strongly inhibited by IL-13 indicating that IL-13 not only affected the phenotype but also the function of human

10 monocytes. Although IL-10 could prevent the IL-13 induced downregulation of CD64 expression, ADCC activity was still inhibited. This supports the notion that ADCC activity is determined by factors other than just the levels of CD64 expression.

IL-13 also affected the expression of FcγRII and FcγRIII. IL-13

15 downregulated the expression of CD32 and CD16 in a dose dependent manner. However, IL-10, but not IFN-γ, could block the IL-13 induced downregulation of CD32 and CD16 on monocytes. These results indicated that the level of Fc receptor expression was highly regulated by cytokines.

20 The only cytokine known to induce the low affinity Fc receptor for IgE (CD23) on monocytes was IL-4. However, IL-13 also induced the expression of CD23 on monocytes. It was demonstrated that the IL-13 induced expression of CD23 could be partially suppressed by IFN-γ. It was also shown that IL-13 could induce

25 production of IgE by PBMC. In addition, IL-13 could initiate germline ε transcription in purified sIgM$^+$ B cells and switching to IgE production when a second signal provided by T cell clones, T cell membranes, or CD40 ligand was present. The production of IgE is regulated by number of cytokines, including soluble CD23, which

30 have either enhancing or inhibitory effects. The effects of IL-13 and IFN-γ on the expression of CD23 by human monocytes fit well within this concept.

35

VI. Activities of IL-4 Antagonist: Interactions

IL-4 and IL-13 are two cytokines secreted by activated T cells which have similar effects on monocytes and B cells. A mutant form of human interleukin-4 (hIL-4) competitively antagonizes both hIL-5 4 and human interleukin-13 (hIL-13). The amino acid sequences of IL-4 and IL-13 are about 30% homologous and circular dichroism spectroscopy (CD) shows that both proteins have a highly α-helical structure. IL-13 competitively inhibits hIL-4-binding to functional human IL-4 receptors (called hIL-4R) expressed on a hIL-4-10 responsive cell line, but not to the cloned IL-4R ligand-binding protein expressed on heterologous cells. hIL-4 has about a 50-fold lower affinity for the IL-4R ligand-binding protein than for the functional IL-4R, while the mutant hIL-4 antagonist protein binds to both receptor types with the lower affinity. The above results 15 demonstrate that IL-4 and IL-13 share a receptor component that is important for signal transduction. In addition, these data establish that IL-4R is a complex of at least two components one of which is a novel affinity-converting subunit that is critical for cellular signal transduction.

20   IL-13 is one of a number of protein hormones called cytokines that are secreted by activated T cells. Human IL-13 elicits morphological and cell-surface phenotype changes on human monocytes and also facilitates growth and immunoglobulin (Ig) production by human B cells. All these biological effects are also 25 elicited by hIL-4, another protein hormone secreted by activated T cells.

The biological actions of IL-4 are mediated by a cell surface receptor that binds IL-4 with high specificity and affinity. [Dissociation constant or $K_d \sim 10^{-10}$ M. See Harada *et al.* in Spits *et* 30 *al.* (eds), *IL-4: Structure and Function*, 1992, CRC Press, Boca Raton, pp. 33-54.] Human and mouse IL-4R have been characterized by cDNA cloning which defined a 130 kDa glycoprotein (herein referred to as IL-4R ligand-binding protein) with a single transmembrane span. The extracellular domain sequence of IL-4R ligand-binding

prot in is structurally homologous to the extracellular domains of other cytokine receptor proteins.

Several of these other proteins participate in heteromeric interactions where one subunit by itself binds the ligand at a
5 relatively low affinity and the other subunit(s) contribute additional binding affinity and often are important for signaling. However, the extracellular domain of the IL-4R ligand-binding protein alone appears to bind IL-4 at the high affinity that characterizes IL-4R on various IL-4-responsive cells. Although the intracellular domain is
10 unimportant for binding, it is important for signal transduction.

The structural homologies between many of the cytokine receptors is mirrored by structural homologies between their ligands. For example, IL-4, interleukin-2 (IL-2), Growth Hormone, Macrophage Colony-Simulating Factor (M-CSF), and Granulocyte
15 Macrophage Colony-Stimulating Factor (GM-CSF) are not related at the sequence level, yet all have a similar compact-core-bundle structure of four antiparallel α-helices. See, e.g., Diederichs *et al.*, *Science* 254:1779 (1991); Bazan, *Science* 57:410 (1992); McKay, *Science* 257:412 (1992); and Powers *et al.*, *Science* 256:1673 (1992).
20 In mouse IL-2, exhaustive mutational analyses led to the discovery that substitution of a residue (Gln141 to Asp) at the C-terminus of the fourth α-helix results in loss of receptor-activation, but retention of most receptor-binding. This mutant protein is a potent and specific competitive antagonist of IL-2 biological action.

25 Based on the structural homology between IL-2 and IL-4, the importance of residues of hIL-4 that might be analogous to mIL-2 Gln141 was investigated. In these experiments, substitution of a residue (Tyr124 with Asp) at the C-terminus of the fourth α-helix of human IL-4 (hIL-4) specifically abrogates IL-4R-activation and
30 renders the protein a competitive antagonist of IL-4 biological action. This property of hIL-4.Tyr124 to Asp (called hIL-4.Y124D) has been described independently. See Kruse *et al.*, *EMBO J.* 11:3237 (1992). This mutant hIL-4 antagonist is defective in interaction with a previously unknown second subunit of the functional IL-4R.
35 In addition, the hIL-4 antagonist blocks many IL-13 biological actions.

Mutagenesis of hIL-4

Based on mutagenesis studies of mIL-2 [Zurawski *et al., EMBO
J. 9*:3899 (1990); Zurawski *et al., EMBO J. 11*:3905 (1992)], the
shared structural frameworks of hIL-2 and hIL-4 [Bazan, *Science
257*:410 (1992); McKay, *Science 257*:412 (1992); Powers *et al.,
Science 256*:1673 (1992)], and assuming evolutionary conservation
of functionally important residues, hIL-4 residues E114, K117, and
Y124 were selected as those most likely to be specifically involved
in receptor activation. Substitution mutagenesis at these positions
used a synthetically reconstructed hIL-4 coding region inserted in
the pTacRBS *Escherichia coli* expression plasmid [Zurawski *et al., J.
Immunol. 137*:3354 (1986)]. Double-stranded synthetic
oligonucleotides (synthesizer and reagents, Applied Biosystems)
corresponding to the sequence between *Sal*I and *Hind*III recognition
sites in the C-terminal coding region and containing equimolar
amounts of each deoxynucleotide at the codon selected for
randomized substitution were ligated to *Sal*I and *Hind*III digested
pTac-hIL-4 plasmid.

Recombinant plasmids were recovered by transformation and
the DNA sequence (Sequenase 2.0 kit, US Biochemical Corp.) of their
*Sal*I-*Hind*III intervals were determined. Partially pure mutant hIL-4
proteins were prepared as described for mIL-2 proteins [Zurawski
et al., EMBO J. 8:2583 (1989)], except that the refolding buffer
contained reduced and oxidized glutathione, and assayed using TF-1
cells. It was found that substitutions at Y124 resulted in proteins
that were partial agonists and that the Y124D substitution had the
most drastic defect in cellular activation. During the course of this
work similar observations were made by Kruse *et al., supra*, who
also showed that hIL-4.Y124D and hIL-4 have similar affinities for
hIL-4R.

For production of pure hIL-4.Y124D, the pTrpC11-hIL-4 *E. coli*
expression plasmid was subjected to PCR (Geneamp kit, Perkin Elmer
Cetus) using the oligonucleotides:
CTCCAAGAACACAACTGAGAAGGAAACCTT (proximal to the single
*Pst*I restriction site in the coding region) and

TTGATTAAGCTT<u>TCAGCTCGAACACTTTGAATCTTTCTC</u> (a *Hind*III
recognition site precedes the underlined part which corresponds to
the C-terminal coding region containing a GAT codon for residue
124). The PCR product and pTrpC11-hIL-4 plasmid were cleaved

5   with *Pst*I and *Hind*III, ligated, and pTrpC11-hIL-4Y124D plasmid
was recovered and validated by transformation and sequence
analysis using previously described methods [Zurawski *et al.*, *EMBO
J.* 8:2583 (1989)].   Corresponding changes in hIL-13 at those
positions should also have IL-13 antagonistic effects.

10

Purification of Proteins

E. coli-derived hIL-4 [van Kimmenade *et al.*, *Eur. J. Biochem.*

15 *173*:109 (1988)], human interleukin-1α (hIL-1α; Kronheim *et al.*,
Bio/Technology 4:1078 (1986), and mIL-13 were purified as
described above.   hIL-4Y124D was prepared from *E. coli* K12 cells
(strain CQ21) harboring the pTrpC11-hIL-4.Y142D plasmid grown
overnight at 37°C in 12 liters of L-Broth containing 50 µg/ml

20 ampicillin in a G 53 rotatory shaker (New Brunswick Scientific) at
200 rpm.   The cells were harvested by centrifugation in a RC-3
centrifuge (all rotors Sorvall) at 4,500 rpm, 10 min, 4°C.   The pellets
were resuspended in 450 ml of TE buffer (50 mM Tris-HCl pH 8,
1 mM EDTA) by shaking at 200 rpm for 15 min.   Cells were ruptured

25 by 4 passes through an ice-cooled Microfluidizer model 110 cell
disrupter (Microfluidics).
Inclusion bodies were collected by centrifugation in a GS-3
rotor at 9,000 rpm, 40 min, 4°C.   The pellet was then washed by
resuspension in 450 ml of TE and Triton X-100 was added to a final

30 concentration of 0.5%.   Samples were kept at room temperature for
30 min and were then pelleted in a GSA rotor at 8,500 rpm, 10 min,
4° C.   The inclusion bodies were resuspended in 60 ml 5 M
guanidine-HCl in PBS (120 mM NaCl, 2.7 mM KCl, 10 mM NaPi pH
7.4), 2 mM reduced glutathione, 0.2 mM oxidized glutathione and

any remaining insoluble material was removed by centrifugation in a SS-34 rotor at 20,000 rpm, 30 min, 4°C.

The supernatant was diluted 10-fold into the same buffer without guanidine hydrochloride and stirred gently overnight at 4°C

5 to permit refolding and oxidization. Concentration and exchange into 100 ml 50 mM Na Acetate pH 5.0 was then performed using a Millipore Pellicon apparatus (Millipore) equipped with a tangential flow ultrafiltration cassette with a size exclusion of 10 kDa. The sample was subjected to anion exchange chromatography (CM

10 sepharose 16/100 column, Pharmacia) in the same buffer with elution via a 0-0.7 M NaCl gradient. Fractions containing hIL-4 protein were pooled and subjected to reverse phase chromatography (Poros R 10/100 column, Perseptive Biosystems) with elution via a gradient of 0-50% acetonitrile in 0.1% trifluoroacetic acid/water.

15 Fractions containing hIL-4 were lyophilized, dissolved in 50 mM Na Acetate pH 5.0, and quantified by densitometry (Molecular Dynamics) of stained SDS-PAGE with chicken egg lysozyme (Sigma) as a standard.

Cell Proliferation Assays

20   Colorimetric cell proliferation assays used the human TF-1 cell line at 30,000 cells per well for 3 days and were performed as described by Mosmann, *J. Immunol. Methods* 65:55 (1983). Cells were assayed in RPMI medium with L-glutamine and 10% fetal bovine serum (JRH Biosciences), 0.5 mM β-mercaptoethanol (Sigma).

25 Cells were maintained in the above medium containing 1 nM hGM-CSF (Schering-Plough).

PHA blasts were prepared by incubation of 10^6 peripheral blood mononuclear cells per ml with 0.1 mg/ml phytohaemagglutinin (Wellcome Diagnostics) in Yssel's medium [see

30 Yssel *et al., J. Immunol. Methods* 72:219 (1984)], supplemented with 1% human AB^{+} serum in 24-well Linbro plates (Flow Laboratories) and were used in the proliferative assay after six days of incubation. SP-B21 is a CD4^{+} cloned T cell line with unknown antigen specificity and was cultured as previously described [Spits *et al., J. Immunol.*

128:95 (1982)]. Proliferative responses of both PHA blasts and SP-B21 cells were determined at 5 x 10⁴ cells per well and were performed and developed colorimetrically after three days as described for TF-1 cells.

5 ## Ligand Binding

Procedures for preparation of cells, separation of bound from free ligand, computer analysis, and quantitation have been described in Zurawski *et al.*, *EMBO J. 11*:3905 (1992). Ba/F3 cells expressing surface hIL-4R-S protein (hIL-4R ligand-binding protein
10 deleted for most of the intracellular domain) were grown as for TF-1 cells except that mouse interleukin-3 (IL-3, 100 U/ml) replaced hGM-CSF and 50 μg/ml gentamycin sulphate (Sigma) and 800 μg/ml Neomycin G418 (Schering-Plough) were added.

^{125}I-radiolabelling of *E. coli*-derived hIL-4 and binding
15 conditions were as described in Harada *et al.*, *J. Biol. Chem. 267*:22752 (1992).

Circular Dichroism Spectroscopy

Secondary structural features of hIL-4, hIL-1α, and mIL-13 proteins were examined on a J720 spectrophotometer with the 450
20 W xenon lamp and J700 data analysis software (Jasco). The samples were dialyzed against 20 mM NaPi, pH 7. Protein concentrations of the samples were re-determined by UV absorption scanning on a Lambda 6 spectrophotometer (Perkin-Elmer). The absorption maximum at 280 nm was used to calculate the amount of protein
25 using theoretical extinction coefficients based on known molecular weights and expected residue absorption contributions. Samples were diluted to 0.2 mg/ml in a 0.2 mm path length cell.

Typical scan parameters for the near UV range were a continuous wavelength scan at 10 mdeg sensitivity, 0.1 nm step
30 resolution at a scan speed of 50 nm/min with a time constant of 2 s. Four accumulations/scan were averaged for an increased signal to noise ratio. Phosphate buffer blanks were run and subtracted out

from subsequent protein scans and the spectra were noise-reduced
using J700 data analysis software.

Mutant hIL-4 Antagonist Blocks IL-13 Action on TF-1 Cells

In a search for mutant hIL-4 antagonists, it was noted that an
5   Asp substitution at residue Tyr124 of hIL-4 resulted in loss of
receptor-activation without significant loss of receptor-binding.   As
expected from these properties,   hIL-4.Y124D was a competitive
antagonist of the action of native hIL-4 on TF-1 cells. TF-1 is a
human pre-myeloid erythroleukemic cell line that shows a growth
10   response to various human protein hormones, such as GM-CSF,
interleukin-3 (IL-3), interleukin-6 (IL-6), IL-4, and both human and
mouse IL-13.   The maximal responses of TF-1 cells to these factors
varies widely, but the maximal biological responses of IL-4 and
IL-13 are similar.   hIL-4.Y124D had no effect on the TF-1 responses
15   to GM-CSF, IL-3, or IL-6.   In contrast, hIL-4.Y124D was a potent
antagonist of both mIL-13 and hIL-13 action on TF-1 cells.
hIL-4.Y124D was equipotent against hIL-4, mIL-13, and hIL-13
activities on TF-1 cells and inhibited in a dose-dependent manner.

IL-13 Competitively Inhibits hIL-4 Binding to TF-1 Cells

20   Since hIL-4.Y124D antagonizes hIL-4 via competitive
inhibition of hIL-4 binding to IL-4R, a similar mechanism was
hypothesized for its action against IL-13.   Such a mode of
hIL-4.Y124D action against IL-13 would imply commonalty between
IL-4R and IL-13R.   This was tested by comparing the abilities of
25   hIL-4 and mIL-13 to competitively displace ^{125}I-hIL-4 binding to
TF-1 cells.   hIL-4 fully competed ^{125}I-hIL-4 binding to TF-1 cells
with the concentration required for 50% inhibition (or IC_{50}) ~ 2 x
10^{-12} M.   mIL-13 also competed ^{125}I-hIL-4 binding.   However,
compared to hIL-4, it could not completely displace ^{125}I-hIL-4
30   binding (~ 70% of the binding was displaced) and its IC_{50} value
(2 x 10^{-10} M) was higher.

<u>IL-13 Does Not Bind to the IL-4R Ligand-binding Protein</u>

A possible basis for the commonalty between IL-4R and
IL-13R is that they are the same. This was tested by comparing the
abilities of hIL-4 and mIL-13 to competitively displace I^{125}-hIL-4
5   binding to a derivative of the cloned hIL-4R ligand-binding protein
expressed on mouse pro-B Ba/F3 cells. Ba/F3 hIL-4R-S cells were
used, which have a large number of binding sites/cell (~ 2000) in
the form of   a hIL-4R ligand-binding protein deleted for most of
the cytoplasmic domain. See Harada *et al.*, *J. Biol. Chem.* 267:22752
10 (1992). Although hIL-4 fully competed ^{125}I-hIL-4 binding to
Ba/F3 hIL-4R-S cells with $IC_{50} \approx 2 \times 10^{-10}$ M, even high levels of
mIL-13 (10^{-6} M) did not compete.

<u>Some hIL-4-responsive Cell Types Do Not Respond to IL-13</u>

The earliest characterizations of the biological activities of
15 IL-13 have shown concordance between cellular responses to IL-4
and IL-13, as described herein. Human peripheral blood
mononuclear cells (PBMNC) activated with phytohaemagglutinin
(PHA) and certain human T cell cloned cell lines such as SP-B21
proliferate in response to hIL-4. Both these hIL-4-responsive cell
20 types did not proliferate in response to hIL-13.

<u>The Binding Properties of hIL-4.Y124D and hIL-4</u>

The binding of hIL-4 to Ba/F3 hIL-4R-S cells ($K_d = 1.6 \times 10^{-10}$ M)
corresponded closely to that previously characterized for the high
affinity IL-4R ($K_d \approx 10^{-10}$ M). Human lymphoma Raji cells have
25 high affinity binding sites for hIL-4 ($K_d \approx 10^{-10}$ M; see Kruse *et al.*,
supra, and hIL-4.Y124D protein binds to these cells with only a 3-
fold reduced affinity compared to hIL-4. hIL-4.Y124D bound
3.5-fold less avidly to the hIL-4 binding sites expressed on Ba/F3
hIL-4R-S cells.

30 TF-1 cells bound hIL-4 with an apparent affinity that was
~50-fold higher than the "high affinity" binding of hIL-4 to Ba/F3
hIL-4R-S cells. This is surprising because although comparisons

were done in parallel and used identical conditions and reagents, these two cell types have been reported to have similar numbers of binding sites and affinities for hIL-4 as defined by equilibrium binding studies. In contrast to the different binding affinities of

5   hIL-4 seen by competitive displacement binding studies, hIL-4.Y124D bound equally to both TF-1 and Ba/F3 hIL-4R-S cells. In other experiments, hIL-4.Y124D was used as the labelled ligand and the results were analogous

IL-4 and IL-13 are Structural Homologues

10   The commonalty between IL-4R and IL-13R prompted a close examination of the sequence relatedness of IL-4 and IL-13. Only the sequences of the mature human and mouse IL-4 and IL-13 proteins were examined, while assuming that known disulfide linkages for IL-4 are preserved for IL-13. There was significant,

15   although low (~30%) sequence homology between IL-4 and IL-13. The significance of this observation was increased when the known structural features of hIL-4 were considered. All of the 25 residues that contribute to the hIL-4 hydrophobic structural core were conserved or had conservative hydrophobic replacements in IL-13.

20   Extensive insertion/deletion differences between IL-4 and IL-13 were, with one exception, confined to loops that connect the four α-helices or two short β-strands. The exception was a shortened α-helix C, although all the α-helix C residues that contribute to the structural core were retained in IL-13.

25   Mouse IL-13, unlike the β-stranded hIL-1α, had a CD absorption spectrum characteristic of a highly α-helical protein such as hIL-4 [see Johnson, *Ann. Rev. Biophys. Chem.* *17*:146 (1988)].

       The similarity in the two cytokines allow for modifications to either cytokine to effect similar properties on the other. Thus,

30   insight into the mechanism of IL-4 antagonist with its receptor will likely be useful in modulating IL-13 with its receptor. In particular, the present study provides locations in the IL-13 molecule which w uld be expected to lead to IL-13 antagonists. Moreover, the described IL-4 receptor would be expected to be modifiable while

retaining its IL-13 antagonist activity. This would suggest that
shortening of the IL-4 antagonist would be useful while retaining its
antagonist function. In particular, specific regions of the cytokine
are suggested as useful to modify to achieve the desired biological
5   activity.

IL-13 and IL-4 Receptors are Functionally Related

The observation that the hIL-4.Y124D antagonist
competitively inhibits the biological action on TF-1 cells of both
hIL-4 and IL-13 demonstrates a relationship between IL-4R and
10   IL-13R. The ability of mIL-13 to compete for ^{125}I-hIL-4 binding
to TF-1 cells confirmed the commonalty of IL-4R and IL-13R. This
relatedness may also have been expected from the similar biological
responses known to be elicited by hIL-4 and IL-13 and perhaps
from the close linkage of the IL-4 and IL-13 genes in both humans
15   and mice. See, e.g., Morgan *et al.*, *Nucleic Acids Res. 20*:5173 (1992),
and other experiments herein. A straightforward explanation of the
above observations would be that IL-4 and IL-13 act through the
same receptor.

However, some cell types responded to IL-4 and not to IL-13.
20   This is *prima facie* evidence that IL-4R and IL-13R are different
entities. This does not exclude the possibility that IL-13 is a weak
partial agonist of IL-4 and that only a subset of IL-4R-bearing cells
can efficiently amplify signals generated by IL-13 binding to IL-4R.
Three lines of evidence helped in resolving the conundrum involving
25   IL-4R and   IL-13R.

Firstly, IL-13 failed to compete for I^{125}-hIL-4 binding to cells
bearing only the hIL-4R ligand-binding protein. This result
demonstrated that the hIL-4R ligand-binding protein is, itself, not
the IL-13R. Secondly, two T cell types responded to hIL-4, but not
30   to hIL-13. If hIL-13 is a partial agonist acting via hIL-4R, then
hIL-13 should competitively antagonize the action of hIL-4 on these
cells. This is not the case in one   hIL-4-responsive T-cell system
that has been tested. Thirdly, if hIL-13 is a partial agonist acting
via hIL-4R, then hIL-13 should be capable of fully competing hIL-4

binding to all cell types bearing hIL-4R. However, IL-13 only partially competed for binding of ^{125}I-hIL-4 to TF-1 cells.

The conclusion from the above three lines of evidence is that IL-13 is not a partial agonist of IL-4 and that IL-4R and IL-13R are

5   different. On TF-1 cells, mIL-13 competed hIL-4 binding and hIL-4.Y124D antagonized the action of IL-13. These data compel a further conclusion that IL-4R and IL-13R have a functionally important receptor component in common. This conclusion is contrary to a consensus that the IL-4R ligand-binding protein itself

10   has all of the functional properties of IL-4R. IL-4R complexity is suggested by studies that find proteins associated with the IL-4R ligand-binding protein. Also, kinetic studies of soluble natural mouse IL-4R ligand-binding proteins indicated that membrane-bound functional IL-4R/IL-4 complexes were more stable than

15   soluble IL-4R/IL-4.

<u>Functional IL-4R Contains an Additional Subunit(s) that Enhances Affinity, Helps Transduce the Signal, and is Shared with IL-13R</u>

Two results from receptor-binding analyses show that IL-4R on TF-1 cells are complex and can exist in a higher affinity state

20   than thought previously. Firstly, the apparent affinity of hIL-4 for hIL-4R on TF-1 cells was ~50-fold greater than for the cloned hIL-4R ligand-binding protein on Ba/F3 IL-4R-S cells. The hIL-4 binding sites on Ba/F3 IL-4R-S cells were the typical 'high affinity' IL-4R that are present on many cell types. Dissociation constant

25   estimates for binding of hIL-4 to hIL-4R commonly vary somewhat, but fall within a 5-fold range of K_d ~10^{-10} M.

Because the experiments were parallel, replicated independently, used the same reagents and cells with similar IL-4R numbers, and gave analogous results using a different labelled

30   ligand, the "higher affinity" hIL-4 binding detected on TF-1 cells should be significant. Secondly, while it bound with only a slightly reduced affinity to IL-4R ligand-binding protein expressed on Ba/F3 cells, hIL-4.Y124D bound to IL-4R on TF-1 cells with an affinity ~50-fold less than did hIL-4. In essence, this result provided an internal

35   c ntrol to confirm the "higher affinity" hIL-4 binding detected on

TF-1 cells. Since the hIL-4R ligand-binding protein cDNA was cloned from TF-1 cells, it is unlikely that an unusual IL-4R ligand-binding protein accounts for the above results.

A model that does account for the above observations   is that
5   functional IL-4R on TF-1 cells are a complex between the IL-4R ligand-binding protein and an additional component (or components) that enhances the affinity of the IL-4R ligand-binding protein for IL-4. This additional component(s) also associates with an IL-13 ligand-binding protein present only on a subset of IL-4-
10   responsive cells to form IL-13R. Furthermore, interaction of hIL-4 Tyr124 residue with this component is essential for productive signal transduction.

Nevertheless, hIL-4.Y124D, which fails to elicit this productive signal transduction, maintains an association between the IL-4
15   ligand-binding protein and this additional component(s). In this model, hIL-4.Y124D antagonizes hIL-4 action by competing for IL-4 binding sites, but antagonizes   IL-13 action by sequestering the additional component(s) from the IL-13R complex by forming a non-productive hIL-4R/hIL-4.Y124D complex.

20   Past Failures to Correctly Define IL-4R and Proposed Tests of the New Model

Two factors may have contributed to past failures to recognize the 'higher affinity' state of IL-4R that were detected on TF-1 cells. Firstly, the integrity of the hIL-4 Tyr124 residue is now known to
25   be vital for this 'higher affinity' binding (this is also the case for the analogous Tyr119 residue of mIL-4 where substitution with Asp resulted in a potent competitive antagonist of mIL-4 biological action). The standard procedure for radio-labelling IL-4 was via iodination of Tyr residues. There are only two Tyr residues in
30   hIL-4, thus it is probable that labelling hIL-4 converted Tyr124 to iodotyrosine.

Indeed, hIL-4.Y124D labelled with the Bolton-Hunter reagent bound about 3-fold less efficiently than hIL-4. It is possible that hIL-4.Y124iodoTyr has a reduced affinity for functional IL-4R and
35   that affinity constants derived using this reagent in direct-binding

experiments have underestimated the actual affinity of IL-4R for IL-4. This was not an issue in the experiments which used hIL-4.Y124iodoTyr as the labelled-ligand and native hIL-4 as a 'cold' competitor. A second factor that may have hindered the discovery of two affinity states for IL-4R is that the difference between the two affinities is only ~50-fold. Thus, if cells have a mixture of IL-4R in both states, or if the 'lower affinity' state predominates, then two affinities may be impossible to recognize separately using conventional methods. The hIL-4R subunit-specific defect of hIL-4.Y124D is a powerful new reagent for dissection of hIL-4R complexity.

The notion that IL-4-responsive cell types vary in IL-4R composition is being tested. Other direct tests of this model will require molecular characterization of the IL-13R ligand binding protein by binding analyses, cross-linking studies, and cloning. However, the reagents to permit direct characterization of IL-13R have not yet been developed. The very low affinity ($K_d \approx 3 \times 10^{-8}$ M) IL-4-binding sites that have been detected on human lymphocytes may be a property of an additional IL-4R component.

Common Subunits in Other Cytokine Receptors

The molecular nature of the functionally important receptor component in common between IL-4R and IL-13R is unclear. The above model to account for our data is based on the existence of other affinity-modulating proteins that are obligatory components shared between several functional cytokine receptors. Such shared components have been discovered in receptors for IL-6, oncostatin-M, leukemia inhibitory factor, and ciliary neurotrophic factor, which all share gp130 [see Kishimoto *et al.*, *Science 258*:593 (1992)], as well as for human IL-3, interleukin-5 (IL-5), and GM-CSF receptors, which all share the β_c protein [Miyajima *et al.*, *Trends In Biochemical Sciences 17*:378 (1992)].

This shared β_c receptor subunit accounts for the observed cross-competition of IL-3, IL-5, and GM-CSF binding to certain cell types. When assayed on TF-1 cells, hIL-4.Y124D did not antagonize

the biological activities of hIL-6, mouse leukemia inhibitory factor, hIL-3, or hGM-CSF, and neither hIL-6 or hGM-CSF competed for hIL-4 binding. Therefore, gp130 or the β_c protein are not likely candidates for the additional IL-4R component, nor the component

5   shared between IL-4R and IL-13R.

On the basis of their common genetic locations/structures and relation in protein structure, it has been proposed that IL-4, IL-3, IL-5, and GM-CSF form a protein family. See Boulay *et al., J. Biol. Chem.* 267:20525 (1992). The biological data regarding commonalty

10   between IL-4 and IL-13 show that IL-13 also belongs to this family. However, the available data support a clear functional separation between the receptors for IL-4/IL-13 and IL-3/IL-5/GM-CSF. For example, no effects of hIL-4.Y124D on IL-3 or GM-CSF responses on TF-1 were noted. Also, in TF-1 cells the pattern of intracellular

15   tyrosine-phosphorylation that is elicited by IL-3/GM-CSF is different from that elicited by IL-4.

Implications of Jointly Antagonizing IL-4 and IL-13 Responses *In Vivo*

The ability of the hIL-4.Y124D antagonist to act against both hIL-4 and hIL-13 biological responses should provoke a reappraisal

20   of the therapeutic potential of hIL-4.Y124D. These results show that, unlike soluble IL-4R ligand-binding protein or anti-IL-4 antibodies, hIL-4.Y124D is not a specific antagonist of hIL-4 action. Inhibitory IL-4 variants have   been suggested as potentially useful drugs in the treatment of IgE-mediated diseases. The possibility

25   exists for antagonizing both hIL-4 and hIL-13 responses by hIL-4.Y124D treatment for various disease states. The structural homology between IL-4 and IL-13 and sharing of receptor subunit(s) between IL-4R and IL-13R suggest that particular IL-13 residues within α-helix D are specifically important for receptor

30   signaling and that substitutions in these residues may result in IL-13 variants that are antagonists. These results also predict that such IL-13 antagonists will be effective antagonists against IL-4-responses on cell types that also respond to IL-13.

Antagonistic Effect on Other IL-13 Activities

Results with cocultures of highly purified B cells and activated T cell clones with 400 U/ml IL-4 showed inhibition of IgE synthesis by the IL-4 antagonist used at 10 μg/ml. See Tables 16 and 17. The assay was as described above for IgE synthesis.

5

10   Table 16: Induction of IgE Synthesis by IL-4, IL-13 and IL-4-Mutant Protein

IgE Synthesis (ng/ml)

Medium	<0.2
IL-4 (200 U/ml)	173±45
IL-13 (200 U/ml)	110±42
IL-4-Antagonist (Y.124, 1 mg/ml)	13±6
Mock-control	<0.2

20

Table 17: IL-4-Mutant Protein Inhibits IL-4-Induced IgE Synthesis by PBMC

IgE Synthesis (ng/ml)

Medium	<0.2
IL-4 (50 U/ml)	265±49
IL-4 (50 U/ml)+Y.124 (0.003 mg/ml)	108±60
IL-4 (50 U/ml)+Y.124 (0.03 mg/ml)	12±5
IL-4 (50 U/ml)+Y.124 (0.3 mg/ml)	12±3
IL-4 (50 U/ml)+Y.124 (3 mg/ml)	5±2
IL-4 (50 U/ml)+Mock-control	194±46

IL-4 antagonists like Y124 also effectively inhibited the proliferation of purified human B cells stimulated by anti--CD40

mAbs in the presence of either IL-4 or IL-13.   IL-13 antagonists
may have similar effects.   Thus, administration of IL-4 and IL-13
antagonists may provide the preferred mehtod not only to inhibit
IgE synthesis, but also to prevent the expansion of IgE producing
5 cells.

VII.   Antibodies to Human IL-13

Rat polyclonal antiserum was raised against *E. coli*-derived
human IL-13 by standard procedures.   See, e.g., Harlow & Lane
10 (1989) or Coligan (1991 and supplements).   Serum from these rats
was useful in immunoprecipitating ^{35}S-methionine labelled
supernatants of COS7 cells expressing IL-13.

Monoclonal antibodies against hIL-13 were produced using
standard methods.   Rats were immunized with *E. coli*-produced
15 hIL-13.   The neutralizing capability of four different monoclonal
antibodies were tested on TF-1 cells stimulated with COS-produced
hIL-13 or *E. coli*-produced hIL-13.   The TF-1 cells (5,000 cells/well)
were incubated for 72 hours with 1:100 dilution of COS-produced
hIL-13 or 5 ng/ml *E. coli*-produced hIL-13, with dilutions for
20 supernatants containing rat anti-hIL-13 Monoclonal antibody.   After
72 hours, cell viability was determined by alamar blue staining.

To determine which monoclonal antibodies from the above
spleen-hybridoma fusions bind to hIL-13 produced from COS or *E.
coli*, an indirect ELISA was performed.   PVC microtier plates were
25 coated for 2 hours with either 0.5 μg/ml *E. coli*-produced hIL-13 or
a 1:15 dilution of COS produced hIL-13 in PBS at 37° C.   A standard
ELISA protocol was used.   Specific binding was observed in both
cases.

Many modifications and variations of this invention can be
30 made without departing from its spirit and scope, as will become
apparent to those skilled in the art.   The specific embodiments
described herein are offered by way of example only, and the
invention is to be limited only by the terms of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5   (i) APPLICANT:
        (A) NAME: Schering Corporation
        (B) STREET: One Giralda Farms
        (C) CITY: Madison
        (D) STATE: New Jersey
10   (E) COUNTRY: U.S.A.
        (F) POSTAL CODE (ZIP): 07940-1000
        (G) TELEPHONE: 201-822-7375
        (H) TELEFAX: 201-822-7039
        (I) TELEX: 219165

15

        (ii) TITLE OF INVENTION: Human   Interleukin-13

        (iii) NUMBER OF SEQUENCES: 6

20   (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: Apple Macintosh
        (C) OPERATING SYSTEM: Macintosh 6.0.5
        (D) SOFTWARE: Microsoft Word 5.1a

25

        (v) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:

30   (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 08/012543
        (B) FILING DATE: 01-FEB-1993

        (vi) PRIOR APPLICATION DATA:
35   (A) APPLICATION NUMBER: US 08/010977
        (B) FILING DATE: 29-JAN-1993

(vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 07/933416
        (B) FILING DATE: 21-AUG-1992

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1290 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TTCGGCATCC GCTCCTCAAT CCTCTCCTGT TGGCACTGGG CCTC ATG GCG CTT TTG 56
                                                  Met Ala Leu Leu
                                                  1

TTG ACC ACG GTC ATT GCT CTC ACT TGC CTT GGC GGC TTT GCC TCC CCA 104
Leu Thr Thr Val Ile Ala Leu Thr Cys Leu Gly Gly Phe Ala Ser Pro
    5               10              15                  20

GGC CCT GTG CCT CCC TCT ACA GCC CTC AGG GAG CTC ATT GAG GAG CTG 152
Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Glu Leu Ile Glu Glu Leu
            25              30              35

GTC AAC ATC ACC CAG AAC CAG AAG GCT CCG CTC TGC AAT GGC AGC ATG 200
Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly Ser Met
            40              45              50

GTA TGG AGC ATC AAC CTG ACA GCT GGC ATG TAC TGT GCA GCC CTG GAA 248
Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala Leu Glu
            55              60              65

TCC CTG ATC AAC GTG TCA GGC TGC AGT GCC ATC GAG AAG ACC CAG AGG 296
Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr Gln Arg
            70              75              80

ATG CTG AGC GGA TTC TGC CCG CAC AAG GTC TCA GCT GGG CAG TTT TCC 344
Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln Phe Ser
85              90              95                  100

AGC TTG CAT GTC CGA GAC ACC AAA ATC GAG GTG GCC CAG TTT GTA AAG 392
Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe Val Lys
                105             110             115

GAC CTG CTC TTA CAT TTA AAG AAA CTT TTT CGC GAG GGA CGG TTC AAC 440
Asp Leu Leu Leu His Leu Lys Lys Leu Phe Arg Glu Gly Arg Phe Asn
                120             125             130
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          TGAAACTTCG AAAGCATCAT TATTTGCAGA GACAGGACCT GACTATTGAA GTTGCAGATT 500

          CATTTTTCTT TCTGATGTCA AAAATGTCTT GGGTAGGCGG GAAGGAGGGT TAGGGAGGGG 560
    5
          TAAAATTCCT TAGCTTAGAC CTCAGCCTGT GCTGCCCGTC TTCAGCCTAG CCGACCTCAG 620

          CCTTCCCCTT GCCCAGGGCT CAGCCTGGTG GGCCTCCTCT GTCCAGGGCC CTGAGCTCGG 680

    10    TGGACCCAGG GATGACATGT CCCTACACCC CTCCCCTGCC CTAGAGCACA CTGTAGCATT 740

          ACAGTGGGTG CCCCCCTTGC CAGACATGTG GTGGGACAGG GACCCACTTC ACACACAGGC 800

          AACTGAGGCA GACAGCAGCT CAGGCACACT TCTTCTTGGT CTTATTTATT ATTGTGTGTT 860
    15
          ATTTAAATGA GTGTGTTTGT CACCGTTGGG GATTGGGGAA GACTGTGGCT GCTGGCACTT 920

          GGAGCCAAGG GTTCAGAGAC TCAGGGCCCC AGCACTAAAG CAGTGGACCC CAGGAGTCCC 980

    20    TGGTAATAAG TACTGTGTAC AGAATTCTGC TACCTCACTG GGGTCCTGGG GCCTCGGAGC 1040

          CTCATCCGAG GCAGGGTCAG GAGAGGGGCA GAACAGCCGC TCCTGTCTGC CAGCCAGCAG 1100

          CCAGCTCTCA GCCAACGAGT AATTTATTGT TTTTCCTCGT ATTTAAATAT TAAATATGTT 1160
    25
          AGCAAAGAGT TAATATATAG AAGGGTACCT TGAACACTGG GGGAGGGGAC ATTGAACAAG 1220

          TTGTTTCATT GACTATCAAA CTGAAGCCAG AAATAAAGTT GGTGACAGAT AAAAAAAAAA 1280

    30    AAAAAAAAAA                                                       1290
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 132 amino acids
  (B) TYPE: amino acid
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Ala Leu Leu Leu Thr Thr Val Ile Ala Leu Thr Cys Leu Gly Gly
1               5                   10                  15

Phe Ala Ser Pro Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Glu Leu
            20              25              30

Ile Glu Glu Leu Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys
                35              40                  45
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```
          Asn Gly Ser Met Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys
                  50              55              60

   5      Ala Ala Leu Glu Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu
                  65              70              75              80

          Lys Thr Gln Arg Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala
                          85              90              95
  10
          Gly Gln Phe Ser Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala
                          100             105             110

          Gln Phe Val Lys Asp Leu Leu Leu His Leu Lys Lys Leu Phe Arg Glu
  15                      115             120             125

          Gly Arg Phe Asn
                  130
  20
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1212 base pairs
  25   (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
  30
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GACAAGCCAG CAGCCTAGGC CAGCCCACAG TTCTACAGCT CCCTGGTTCT CTCACTGGCT   60

CTGGGCTTC ATG GCG CTC TGG GTG ACT GCA GTC CTG GCT CTT GCT TGC        108
          Met Ala Leu Trp Val Thr Ala Val Leu Ala Leu Ala Cys
  35       1               5                   10

CTT GGT GGT CTC GCC GCC CCA GGG CCG GTG CCA AGA TCT GTG TCT CTC      156
Leu Gly Gly Leu Ala Ala Pro Gly Pro Val Pro Arg Ser Val Ser Leu
        15              20                  25
  40
CCT CTG ACC CTT AAG GAG CTT ATT GAG GAG CTG AGC AAC ATC ACA CAA      204
Pro Leu Thr Leu Lys Glu Leu Ile Glu Glu Leu Ser Asn Ile Thr Gln
  30              35                  40                  45

  45   GAC CAG ACT CCC CTG TGC AAC GGC AGC ATG GTA TGG AGT GTG GAC CTG   252
       Asp Gln Thr Pro Leu Cys Asn Gly Ser Met Val Trp Ser Val Asp Leu
                       50              55                  60

       GCC GCT GGC GGG TTC TGT GTA GCC CTG GAT TCC CTG ACC AAC ATC TCC   300
  50   Ala Ala Gly Gly Phe Cys Val Ala Leu Asp Ser Leu Thr Asn Ile Ser
                       65              70                  75
```

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AAT TGC AAT GCC ATC TAC AGG ACC CAG AGG ATA TTG CAT GGC CTC TGT    348
Asn Cys Asn Ala Ile Tyr Arg Thr Gln Arg Ile Leu His Gly Leu Cys
            80                  85                  90

AAC CGC AAG GCC CCC ACT ACG GTC TCC AGC CTC CCC GAT ACC AAA ATC    396
Asn Arg Lys Ala Pro Thr Thr Val Ser Ser Leu Pro Asp Thr Lys Ile
        95                 100                 105

GAA GTA GCC CAC TTT ATA ACA AAA CTG CTC AGC TAC ACA AAG CAA CTG    444
Glu Val Ala His Phe Ile Thr Lys Leu Leu Ser Tyr Thr Lys Gln Leu
110                 115                 120                 125

TTT CGC CAC GGC CCC TTC TAATGAGGAG AGACCATCCC TGGGCATCTC          492
Phe Arg His Gly Pro Phe
                130

AGCTGTGGAC TCATTTTCCT TTCTCACATC AGACTTTGCT GGGGAGAGGC AGGGAGGAGG   552

GTTGAGGAGG AAGGGAGATG CCTCAGCTTT GGCCTCAGCC TGCACTGCCT GCCTAGTGCT   612

CAGGGTCTCA GCCTGGCAAC ACCCCCACCC CACCCCCACC CCCGCCGCCC CATCCCATCC   672

CTACAGAAAA CTGCAGCAAG ACCGTGAGTC CAGCCTGTGG CCTGGTCCAC ACAGGGCAAC   732

TGAGGCAGGC AGCAGCTTGA GCACATTTCT TCTTGATCTT ATTTATTATG GTTGTGTGTT   792

ATTTAAATGA GTCTGTCAGT ATCCCGGTGG GGACATGGTT TGCTGCCTAT GCCCTGGGGG   852

CTCCAGCATT GAAGCAGTGG GCTCTGGGGT CCCTGGCAAT ATTACTGTAT ACATAACTCT   912

GCTACCTCAC TGTAGCCTCC AGGTCTCACC CCAGGCAGGA GATGGGAGGG GAGGCCAGAG   972

CAACACTCCT GTCTGCCACG GCAGCAACCA GCCCTCAGCC ATGAAATAAC TTATTGTTTT  1032

GTTCTTATAT TTAAAGTATT AAATAGCTTA GCAAAGAGTT AATAATATAT GGAAGAATGG  1092

CCTGTTACAC TCAAGGTGAT GTGTAGTGAA TGGGGGGAGG GTGGTGGGTT TGTCACTGAA  1152

CAAACTTTTC ATTGACTGTC AAACTAGAAA CCGGAAATAA AGATGGTGAC AGATAAAAAA  1212
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(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 131 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Ala Leu Trp Val Thr Ala Val Leu Ala Leu Ala Cys Leu Gly Gly
 1               5                10                15

Leu Ala Ala Pro Gly Pro Val Pro Arg Ser Val Ser Leu Pro Leu Thr
            20              25              30

Leu Lys Glu Leu Ile Glu Glu Leu Ser Asn Ile Thr Gln Asp Gln Thr
        35              40              45

Pro Leu Cys Asn Gly Ser Met Val Trp Ser Val Asp Leu Ala Ala Gly
     50              55              60

Gly Phe Cys Val Ala Leu Asp Ser Leu Thr Asn Ile Ser Asn Cys Asn
 65              70              75              80

Ala Ile Tyr Arg Thr Gln Arg Ile Leu His Gly Leu Cys Asn Arg Lys
            85              90              95

Ala Pro Thr Thr Val Ser Ser Leu Pro Asp Thr Lys Ile Glu Val Ala
            100             105             110

His Phe Ile Thr Lys Leu Leu Ser Tyr Thr Lys Gln Leu Phe Arg His
            115             120             125

Gly Pro Phe
        130
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30   (2) INFORMATION FOR SEQ ID NO:5:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 35 base pairs
          (B) TYPE: nucleic acid
35   (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

40   ACAGCTCGAG CCATGGTGTC TTTGCCTCGG CTGTG   35

     (2) INFORMATION FOR SEQ ID NO:6:

     (i) SEQUENCE CHARACTERISTICS:
45   (A) LENGTH: 36 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTAGCTCGAG CTCACCGGGA CTTTAAACCA CAGATG 36

5

<u>WHAT IS CLAIMED IS</u>:

    1. Human IL-13 having an amino acid sequence defined by SEQ ID NO: 2.

    2. A pharmaceutical composition comprising a
10 pharmaceutically acceptable carrier and an effective amount of human IL-13 having an amino acid sequence defined by SEQIDNO.2.

    3. A method for making a pharmaceutical composition comprising admixing a pharmaceutically acceptable carrier and an effective amount of human IL-13 having an amino acid sequence
15 defined by SEQ ID NO: 2.

    4. An antibody, preferably a monoclonal antibody, against human IL-13 having an amino acid sequence defined by SEQ ID NO: 2.

    5. A pharmaceutical composition for inhibiting antibody
20 isotype switching to IgE or IgG4 comprising a pharmaceutically acceptable carrier and an effective amount of an antagonist of IL-13.

    6. A method for making a pharmaceutical composition for inhibiting antibody isotype switching to IgE or IgG4 comprising admixing a pharmaceutically acceptable carrier and an effective
25 amount of an antagonist of IL-13.

    7. Use of an antagonist of IL-13 for inhibiting antibody isotype switching to IgE or IgG4.

    8. Use of an antagonist of IL-13 for the manufacture of a pharmaceutical composition for inhibiting antibody isotype
30 switching to IgE or IgG4.

9.   The pharmaceutical composition, method or use of any one of claims 5 to 8 in which the antagonist is an antibody against IL-13, preferably a monoclonal antibody.

10.   The pharmaceutical composition, method or use of any one of claims 5 to 9 in which the inhibition of antibody isotype switching produces reduced levels of IgE antibodies.

11.   The pharmaceutical composition, method or use of any one of claims 5 to 10 in which the antagonist is an antagonist of human IL-13.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/24 C12P21/02 C07K13/00 A61K37/02 C12P21/08
A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP,A,0 506 574 (ELF SANOFI) 30 September 1992 see the whole document especially figures 2 and 3 , pages 43-44 and the claims ---	1-4
A	JOURNAL OF IMMUNOLOGY vol. 142, no. 2 , 15 January 1989 , BALTIMORE US pages 679 - 687 K. D. BROWN ET AL 'a family of small inducible proteins secreted by leukocytes are members of a new super family that includes leukocyte and fibroblast-derived inflammatory agents, growth factors and indicators of various activation processes' see the whole document ---	1

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 1993	16. 12. 93
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Le Cornec, N

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No

PCT/US 93/07645

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 90 , April 1993 , WASHINGTON US pages 3735 - 3739 A.N.J. MCKENZIE ET AL 'Interleukin 13 , a T-cell-derived cytokine that regulates human monocyte and B-cell function' see the whole document ---	1-4
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 90 , April 1993 , WASHINGTON US pages 3730 - 3734 J. PUNNONEN ET AL 'Interleukin 13 induces Interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells' see the whole document ---	1-4
P,X	EMBO JOURNAL vol. 12, no. 7 , July 1993 , EYNSHAM, OXFORD GB pages 2663 - 2670 S. M. ZURAWSKI ET AL 'Receptors for Interleukin-13 and Interleukin-4 are complex and share a novel component that functions in signal transduction' see the whole document -----	1-3,5-8, 10,11

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0506574	30-09-92	FR-A- 2674537	02-10-92
		FR-A- 2685919	09-07-93
		WO-A- 9217586	15-10-92

Docket N . P31005C6
(predecess r SN: 09/599,074)

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